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14. ABSTRACT Biomonitoring of exposure to the insecticide permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA). However, urinary metabolites have the disadvantage that they are rapidly excreted. We are engaged in the development of a methodology to assess the cumulative internal dose of exposure to permethrin, which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans-Cl2CA) will form persistent adducts to proteins, in analogy with the glucuronide conjugates of structurally related drugs. The 3-PBA and Cl2CA glucuronide metabolites of permethrin have been successfully chemically and enzymatically synthesized. The reactivity of these metabolites with various amino acids, peptides and albumin has been studied; various distinct adducts could be identified by LC tandem mass spectrometry. Subsequently, a method was developed for analysis of albumin adducts of the glucuronides in plasma, which is based on pronase digestion of albumin followed by LC-tandem MS analysis of the lysine adducts. For 3-PBA glucuronide, it was attempted to quantify adduct formation by using [14C] labelled 3-PBA glucuronides, obtained by combined chemical and enzymatic synthesis. From a 2-D gel electrophoresis experiment with [14C]labelled 3-PBA glucuronide and human plasma, it could be concluded that the major binding site was human serum albumin. In case of liver homogenates only low-molecular radioactive material could be detected, indicating that hydrolysis or binding to low-molecular material predominated. In a preliminary animal experiment the acyl adduct of 3-PBA to lysine could be detected, even 22 days after the exposure. In plasma samples of humans who had worn permethrin-treated suits, however, none of the adducts could be detected. Although it seems that the 3-PBA glucuronide adducts are not being formed in such amounts that they can serve as biomarkers for permethrin exposure within the framework of biomonitoring studies, the current large scale use of permethrin warrants further investigations towards the toxicological relevance of protein binding by the reactive glucuronide metabolites.

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SUMMARY

Biomonitoring of exposure to the insecticide permethrin is usually performed by analysis of its urinary metabolite 3-phenoxybenzoic acid (3-PBA). However, urinary metabolites have the disadvantage that they are rapidly excreted. We are engaged in the development of a methodology to assess the cumulative internal dose of exposure to permethrin, which is based on the assumption that (reactive) glucuronide conjugates of the major permethrin metabolites 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans-Cl_2CA) will form persistent adducts to proteins, in analogy with the glucuronide conjugates of structurally related drugs. The 3-PBA and Cl_2CA glucuronide metabolites of permethrin have been successfully chemically and enzymatically synthesized. Their identities have been assessed by means of ^{1}H- NMR spectroscopy and LC tandem mass spectrometry. The reactivity of these metabolites with various amino acids, peptides and albumin has been studied; various distinct adducts could be identified by LC tandem mass spectrometry. Subsequently, the adduct formation of the glucuronides of 3-PBA and Cl_2CA was studied in plasma. A method was developed for analysis of albumin adducts of the glucuronides, which is based on pronase digestion of albumin followed by LC-tandem MS analysis of the lysine adducts. For 3-PBA glucuronide, it was attempted to quantify adduct formation by using [^{14}C] labelled 3-PBA glucuronides, obtained by combined chemical and enzymatic synthesis. Quantitation was thwarted due to non-covalent association of the glucuronides to the proteins. From a 2-D gel electrophoresis experiment with [^{14}C]labelled 3-PBA glucuronide (with a high specific activity), it could be concluded that the major binding site in human plasma was human serum albumin. In case of liver homogenates only low-molecular radioactive material could be detected, indicating that hydrolysis or binding to low-molecular material predominated. In a preliminary animal experiment in which the animal was exposed to 20 mg/kg permethrin, the acylation adduct of 3-PBA to lysine could be detected, even after 22 days. In plasma samples of humans who had worn permethrin-treated suits, however, none of the adducts could be detected. Although it seems that the 3-PBA glucuronide adducts are not being formed in such amounts that they can serve as biomarkers for permethrin exposure within the framework of biomonitoring studies, the current large scale use of permethrin warrants further investigations towards the toxicological relevance of protein binding by the reactive glucuronide metabolites.

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LIST OF ABBREVIATIONS

Boc	tert-butyloxycarbonyl
Cl_2CA	3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
Diad	Diisopropyl azodicarboxylate
DIPEA	diisopropyl ethylamine
DMF	*N*,*N*-Dimethylformamide
EDTA	ethylenediaminetetraacetic acid
EtOH	Ethanol
Fmoc	fluorenylmethyloxycarbonyl
HATU	*O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HOBT	N-hydroxybenzotriazole
MeOH	methanol
NMM	N-methylmorpholine
NSAID	Non-Steroidal Anti-Inflammatory Drugs
3-PBA	3-phenoxybenzoic acid
$Pd(PPh_3)_4$	Tetrakis(triphenylphosphine)palladium(0)
PPh_3	Triphenylphosphine
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
TFA	trifluoroacetic acid
THF	Tetrahydrofuran
TIS	tri-isopropylsilane
TLC	thin layer chromatography
UDP	uridine diphosphate
Z-Lys	N-α-Benzyloxycarbonyl-L-lysine

I INTRODUCTION

The pyrethroid permethrin is one of the most widely used insecticides. It is effective in the control of ticks, mites and lice, while having little adverse effects in humans. Its toxicity in insects, and in humans, is based on binding to sodium channels in the nervous system, leading to prolongation of the depolarizing after-potential, repetitive after-discharges and hyperexcitation (Narahashi, 2002). Recently, it has been argued that voltage-sensitive calcium channels may also be targets of pyrethroid action (Shafer and Meyer, 2004). Permethrin has been used extensively by the allied troops in the Gulf War and in operation Iraqi Freedom, *e.g.*, by impregnating it into battle dress uniforms and bed nettings. In this way permethrin can be absorbed through the skin, while oral and respiratory exposure can also occur. Probably, soldiers can be exposed to rather high doses of permethrin by migration of the compound from clothing to the skin surface (see, *e.g.*, Snodgrass, 1992). Although permethrin is generally considered as a rather safe compound, a number of adverse effects have been reported. Occupationally exposed people have been reported to experience facial skin sensations. Symptoms of acute poisonings include dizziness, headache, nausea, anorexia, and fatigue. In case of heavy exposures, muscle fasciculation and altered consciousness have been reported (He et al, 1989; Aldridge, 1990). It has been shown in animal experiments that combined exposure to (high dosages) of permethrin, DEET and pyridostigmine bromide resulted in enhanced neurotoxicity, increased mortality, increased oxidative stress, and behavioral alterations (Abou-Donia et al., 1996; Abu-Qare and Abou-Donia, 2000a, 2001; Abdel-Rahman et al, 2004; for a review see Abu-Quare and Abou-Donia, 2003). On the basis thereof it was postulated that such combined exposures might have contributed to the etiology of the so-called Gulf War Illness (e.g., Jamal et al, 1998). Therefore, careful biomonitoring of exposure to permethrin is important for the military community.

The metabolism of permethrin has been investigated in several species (Huckle et al, 1981). The major metabolites are cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (cis/trans Cl_2CA) and 3-phenoxybenzoic acid (3-PBA; see e.g., Tyler et al., 2000; Hardt & Angerer, 2003). The latter metabolite is formed in three phases (see Figure 1). First, esterase-mediated cleavage of the parent compound will give 3-phenoxybenzyl alcohol, while in the second phase this compound is oxidized enzymatically through an intermediate aldehyde (Bast and Kampffmeyer, 1998; Heder et al, 2001; Choi et al, 2002) to 3-PBA. The 4'-hydroxy derivative of 3-PBA has been identified as the major metabolite of permethrin in the rat (Angerer and Ritter, 1997). Subsequently, phase II metabolism will give the respective conjugates, mostly glucuronides, which facilitate the excretion process. The role of human carboxylase enzymes in permethrin hydrolysis has recently been examined by Ross and coworkers ((Ross and Crow, 2007; Crow et al, 2007).

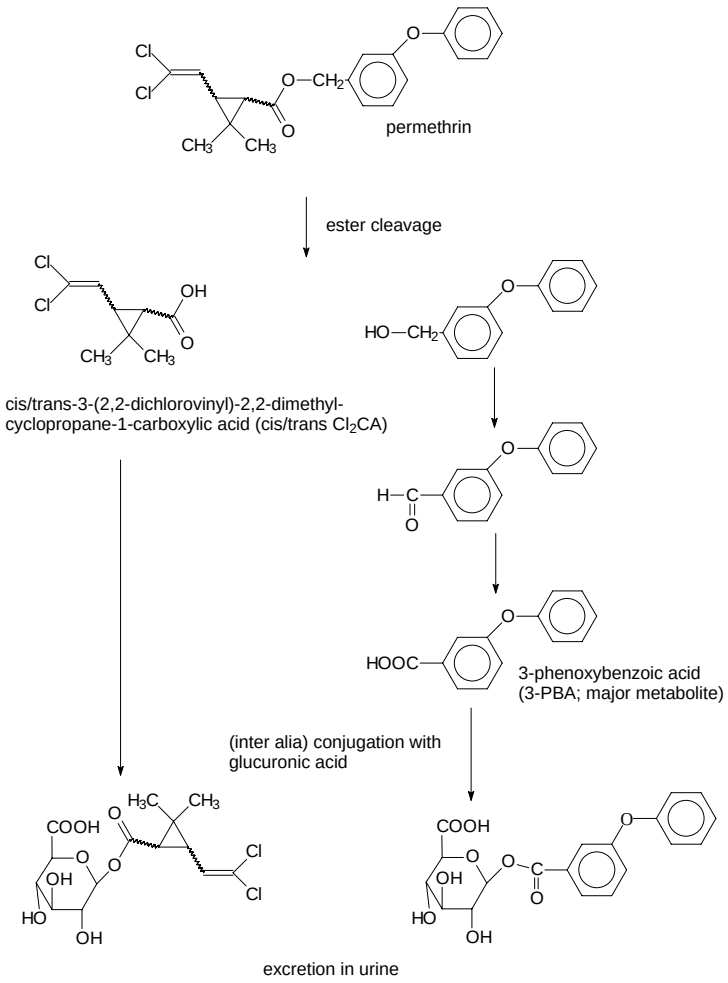


Figure 1. Metabolism of permethrin in mammals.

Biomonitoring of exposure to permethrin is usually performed by analysis of its urinary metabolite 3-PBA, after acidic hydrolysis of its glucuronide (see, e.g., Hardt and Angerer, 2003; Leng et al., 2003; Abu-Qare and Abou-Donia, 2000b; Baker et al, 2004), albeit that conjugates of cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid are excreted into human urine in similar quantities as the PBA derivatives (Hardt and Angerer, 2003). Studies with a volunteer who had been exposed (orally) to the closely related pyrethroid cyfluthrin revealed that most (93%) of the urinary metabolites are excreted within 24 h (Leng et al., 1997). Consequently, urine samples for biomonitoring purposes should be taken within the first 24 h after exposure. More persistent biomarkers should surpass this drawback. This observation constitutes an evident research gap.

It is well known that protein adducts of xenobiotics represent a much more persistent class of biomarkers than metabolites excreted into urine, having half lives up to several weeks or months. For instance, protein adducts in human tissues have provided mechanistic insight into the epidemiological associations between smoking and cancer (Phillips, 2002). In our laboratory we developed methods for biomonitoring of exposure to CW agents based on mass spectrometric analysis of such protein adducts, , e.g., adducts of sulfur mustard with hemoglobin and albumin and of adducts of nerve agents with butyrylcholinesterase (for overviews see Black and Noort, 2005 and Noort et al., 2002, 2005). In view of its chemical structure, it should not be expected that permethrin itself will react with proteins to give adducts, as experimentally established for albumin (Abu-Qare and Abou-Donia, 2002).

In the current study we explore the feasibility of biomonitoring of exposure to permethrin based on the determination of long-lived protein adducts derived from metabolites of permethrin. Within this context we use the (presumed) protein adducts of glucuronides of the two major carboxylic acid metabolites of permethrin, i.e., 3-PBA and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (see Figure 1). Such adducts may serve as cumulative biomarkers for chronic exposure to permethrin, since
the O-acyl glucuronides represent a unique class of electrophilic metabolites, capable of reaction with nucleophilic sites in proteins. Numerous examples of these reactions have been documented in which the O-acyl glucuronides originated from drugs having a carboxylic acid moiety, such as several non-steroid anti inflammatory drugs (NSAID's), lipid lowering agents (gemfibrozil, clofibric acid), diuretic agents (furosemide)and the antiepileptic drug valproic acid (Benet et al, 1993; see Bailey and Dickinson, 2003 for an extensive overview). McKinnon and Dickinson (1989) investigated the persistence of adducts of diflunisal- and probenecid-glucuronides with plasma proteins in volunteers. The adducts were still measurable at least one month after the parent drugs were undetectable. These results hold promise for the current project.

Statement of work

The work described here is focused on the development of methods for biomonitoring exposure to permethrin, which are based on long-lived adduct with proteins. This will enable biomonitoring of chronic, low-level exposures to this compound. In order to develop such methods:

1. It will be assessed whether the potentially reactive permethrin metabolites 3-phenoxybenzoic acid glucuronide (3-PBA glucuronide) and cis/trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid glucuronide (cis/trans-Cl_2CA glucuronide) can form adducts with proteins in human plasma.

2. A sensitive liquid chromatography tandem mass spectrometry procedure will be developed for the most suitable/abundant adduct with albumin, after enzymatic digestion.

3. The in vivo formation of the adduct will be further evaluated in laboratory animals. It will also be evaluated whether the adduct can be analyzed in plasma samples of US soldiers who have used permethrin extensively during deployment.

Time schedule
Year 1: 1
Year 2: 2
Year 3: 3

II MATERIALS AND INSTRUMENTATION

II.1 Materials

The following commercially available products were used:

3-Phenoxybenzoic acid (Fluka, Zwijndrecht, The Netherlands); Cl_2CA (Specs Research Laboratory, Delft, The Netherlands). Acetonitrile (Baker Chemicals, Deventer, The Netherlands); pronase Type XIV from Streptomyces Griseus (E.C. 3.4.24.31) (Sigma Chemical Co., St. Louis, MO, U.S.A.); benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) and Fmoc-amino acids (Novabiochem); trifluoroacetic acid, trypsin (Aldrich, Brussels, Belgium). Protease inhibitor cocktail tablets (Complete Mini) purchased from Roche Diagnostics (Mannheim, Germany). Coomassie blue and the RC/DC protein assay were obtained from BioRad (Veenendaal, The Netherlands). Protein concentration was determined with the RC/DC protein assay (BioRad).

Slyde-A-Lyzer cassettes were obtained from Pierce. Centrex UF-2 10 kDa filters were obtained from Schleicher & Schuell (Dassel, Germany). Ultrafree (100 kD molecular weight cut-off; 15 ml) centrifugal ultrafilters were obtained from Millipore (Bedford, MA). Albumin affinity chromatography was carried out on HiTrap Blue HP columns (1 ml; Amersham Biosciences, Uppsala, Sweden). Desalting of albumin fractions was carried out on PD-10 columns containing Sephadex G-25 (Amersham Biosciences, Uppsala, Sweden).

II.2 Instrumentation/devices

Solid phase peptide synthesis was carried out on a Syro 2000 (Multisyntech, Germany) peptide synthesizer on a 10 µmol scale, using commercially available amino acids and customized Fmoc-based protocols. After synthesis, the peptides were split off from the resin and purified to homogeneity with semi-preparative HPLC.

Liquid chromatography experiments were run on an ÁKTA explorer chromatography system (Amersham Pharmacia, Uppsala, Sweden). Columns used were a Pep RPC 5/5 column (Pharmacia, Uppsala, Sweden), a Zorbax SB C-18 column (4.6 mm x 150mm; 5 µm, Zorbax, Mac-Mod Analytical, Chadds Ford, PA, USA) and a Source 15 RPC column (Amersham Pharmacia, Uppsala, Sweden).

LC/electrospray tandem mass spectrometric analyses for obtaining structural information were conducted on a Q-TOF hybrid instrument equipped with a standard Z-spray electrospray interface (Micromass, Altrincham, UK) and an Alliance, type 2690 liquid chromatograph (Waters, Milford, MA, USA). The chromatographic hardware consisted of a pre-column

splitter (type Acurate; LC Packings, Amsterdam, The Netherlands), a sixport valve (Valco, Schenkon, Switzerland) with a 10 or 50 µl injection loop mounted and a PepMap C_{18} (LC Packings) or Vydac C18 column (both 15 cm x 300 µm I.D., 3 µm particles). A gradient of eluents A (H_2O with 0.2% (v/v) formic acid) and B (acetonitrile with 0.2% (v/v) formic acid) was used to achieve separation. The flow delivered by the liquid chromatograph was split pre-column to allow a flow of approximately 6 µl/min through the column and into the electrospray MS interface. MS/MS product ion spectra were recorded using a cone voltage between 25 and 40 V and a collision energy between 30 and 35 eV, with argon as the collision gas (at an indicated pressure of 10^{-4} mBar).

Other mass spectrometric analyses were carried out on a TSQ Quantum Ultra mass spectrometer (Finnigan, Thermo Electron Corporations, San Jose, USA) equipped with an Acquity Sample Manager and Binary Solvent Manager (Waters, Milford, USA). For LC-MS experiments, the liquid chromatograph was connected to the mass spectrometer source via the Sample Manager equipped with a 10 µl loop and an Acquity BEH C18 column (1.7 µ particles, 1 x 100 mm; Waters, Milford, USA). The liquid chromatography system was run with a 25 minute linear gradient from 100% A to A/B 55.5/45.5 v/v (A: 0.2% formic acid in water; B: 0.2% formic acid in acetonitrile) at a flow rate of 0.09 ml/min. The TSQ Quantum Ultra mass spectrometer was operated with a spray voltage of 3 kV, a source CID of 0 V, a sheath gas pressure of 41 A.U., aux gas pressure of 2 A.U. and a capillary temperature of 350 ºC. Positive electrospray product ion spectra were recorded at an indicated collision energy of 15-20 eV, using argon as the collision gas at a pressure of 1.5 mTorr. Negative electrospray product ion SRM data was recorded at an indicated collision energy of 15-20eV.

^{1}H-NMR spectra were recorded on a Varian (Palo Alto, CA, U.S.A.) VXR 400S spectrometer operating at 400.0 MHz respectively. Chemical shifts are given in ppm relative to tetramethyl silane. The solvent signals at 2.525 ppm (residual Me_2SO-d_5 in Me_2SO-d_6) or 7.260 ppm (residual $CHCl_3$ in $CDCl_3$) served as a reference.

Radio-HPLC analyses were performed using a series 200 HPLC pump, UV/Vis detector and Radiomatic 625 TR Flow scintillation Analyzer (all by Perkin Elmer, Shelton, CT).

Radioactivity was counted using a tri-Carb 2900 TR liquid scintillation analyzer (Packard Instrument Co., Downers Grove, Il). Liquid Scintillation Cocktail used was Hionic Fluor (PE, Shelton, CT).

III EXPERIMENTAL PROCEDURES

Allyl α/β-D-Glucopyranuronate (1)

To a solution of D-glucuronic acid (4 g, 20.6 mmol) in 40 ml of DMF was added DBU (3.4 ml, 22.6 mmol) at 25ºC. The mixture was stirred for 15 minutes, and subsequently allyl bromide (2.2 ml, 24.6 mmol) was added. The mixture was stirred over the weekend at room temperature. The solvent was then removed under high vacuum and heating. Portions of the product were purified before use in subsequent reactions by chromatography over silica gel (5-20% MeOH/DCM) and washed with DCM to remove traces of DBU.

Yield: 2.6 g of a white powder (53%). MS (ES^-) data: 279 [M + $HCOO^-$]

Allyl 1-O-(3-phenoxybenzoyl) α/β-D-glucopyranuronate (2)

To a solution of 3-phenoxybenzoic acid (473 mg, 2.04 mmol) and triphenylphosphine (540 mg, 2.04 mmol) in THF (8 ml) and DMF (1 ml) at -10ºC was added DIAD (400 µl, 2.04 mmol). After 5 min, a solution of compound (1) (170 mg, 1.04 mmol) in THF (2 ml) and DMF (0.5 ml) was added slowly over 10 minutes. After 5 h the solvent was removed under vacuum and the product purified over silica gel (0-10% EtOH/DCM). This yielded the desired product (2) as an enantiomeric mixture.

Yield 29.4 mg (9.8%) of an amorphous solid. MS (ES^-) data: 475 [M + $HCOO^-$], 213 (3-PBA). ^{1}H-NMR: H1, β-anomer (δ 5.7, 1H, d, J=7.74Hz); H1, α-anomer (δ 6.3; 1 H, d J=3.5Hz).

1-O-(3-phenoxybenzoyl) β-D-glucopyranuronic acid (3) (3-PBA glucuronide), first batch

To a solution of allyl ester (2) (23.6 mg, 0.05 mmol) in THF (100µl) at 0ºC was added $Pd(PPH_3)_4$ (5.8 mg, 0.005mmol) followed by pyrrolidine (4.3 µl/50 µl THF). The mixture was stirred for 60 minutes at this temperature, and when the reaction was judged to be complete as monitored by TLC, the solvent was removed under vacuum. The product was purified by preparative HPLC and yielded the β-3-PBA glucuronide as an amorphous solid (1.1 mg, 5.6%) The β-form of the product was confirmed by ^{1}H-NMR (δ 5.7, 1H, d, J=7.74Hz), with no traces of α-anomer detectable. The correct mass was assessed by LC-tandem (ES-)MS: 389, $[M-H]^-$, 503 $[M+TFA]^-$.

Allyl 1-O-(3-phenoxybenzoyl) β-D-glucopyranuronate (2), second batch; preparation by by selective acylation

3-PBA, allyl glucuronate (1) and HATU were stirred in dry acetonitrile with NMM under nitrogen at room temperature. After 1 h, the solvent was removed under vacuum and coevaporated with DCM/pentane. The product was purified over silica gel (0-10%

EtOH/DCM). This yielded the desired product as a single isomer (according to HPLC; gradient elution 5-80% CH_3CN/H_2O + 0.1%TFA in 40 min). Yield: 1.37g, 80%.

1-O-(3-phenoxybenzoyl) β-D-glucopyranuronate (3), second batch

The deprotection of this batch to obtain 3-PBA acyl glucuronide **(5)** was performed as described above with the following quantities. Allyl ester (2) (810 mg, 1.88 mmol) $Pd(PPh_3)_4$ (293 mg, 0.188 mmol) and pyrrolidine (150 µl, 1.80 mmol) in 8 ml dry THF at 0ºC. After 2h, when the reaction was judged complete by HPLC, the solvent was removed under vacuum and purified over silica gel (5-50% EtOH/DCM). This yielded the product as a powder (128 mg, 17%) HPLC indicated this batch as a single isomer, also when spiked with the 3-PBA acyl glucuronide from the first synthesis batch using the other method. LC-MS(MS) confirmed the mass $[M-H]^-$ = 389 and also showed a single peak in the total ion current.

^{1}H NMR spectroscopy showed some serious disturbances in the chemical shift region (around 5.7) where the characteristic H1-β doublet is located. This interference can be caused by solid particles in the solution or the presence of remains of allylic compounds, which is probably the source of the signals at 5.95, 5.53 and 5.48. No traces of the α-anomer were observed.

Allyl-1-O-(E/Z-3-(2,2-dichlorovinyl)-2,2-dimethyl-cyclopropanoyl-β-D-glucopyranuronate (4)

Reaction of Cl_2CA (mixture of cis/trans; 51 mg), allyl glucuronate (55 mg) and HATU (90 mg) was carried out as described for compound **2**. The desired compound **4** was isolated by means of silica gel column chromatography. According to ^{1}H-NMR spectroscopy, both the α- and β-anomer were present, which could not be separated by silica gel column chromatography. Since the starting material Cl_2CA consisted of the cis and trans isomers, there are actually two α- and β-anomers, with the characteristic shifts and coupling constants. For the α-anomer: δ 6.2 and J = 3.5 Hz and for the β-anomer: δ 5.8 and J = 7.8 Hz. Between 5.9 and 6.0 the allyl multiplet was visible. With HPLC analysis two very closely-eluting peaks were visible, that could not be separated.

Mass spectrometric data (ES-): 469 and 471 [M + HCOO-]; the two chloro-isotopes were clearly visible.

1-O-(E/Z-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropanoyl-α/β-D-glucopyranuronate (5)

Deprotection of compound **4** was performed as was described for compound, with the exception that the initial reaction temperature was – 80 ºC. The temperature was slowly increased to 0 ºC when the reaction components were thoroughly mixed. According to MS, deprotection was complete after 1 h.

Mass spectrometric data (ES-): 382.9 [M-H]-, 768.9 [2M-H]-.

Incubations of 3-PBA acyl glucuronide with model amino acids and peptides

Incubations (1 ml total volume) of 3-PBA glucuronide (100μM) were performed in potassium phosphate buffer (0.05M, pH 7.4) at 37ºC, for 3 h, in the presence of one of the model compounds (10 mM), Z-Lys-OH, glutathione, ASSAKQR or LKZASLQK. In addition, incubations were performed in absence of the compound or of 3-PBA glucuronide, in order to serve as control sample. Samples of the incubation mixtures were taken at different times and immediately measured by LC-MS.

Incubation of Cl_2CA glucuronide with glutathione and Z-Lys-OH

Incubation of Cl_2CA glucuronide with glutathione and Z-Lys-OH was carried out as described for 3-PBA glucuronide (see above). Only in case of glutathione, the expected adduct could be identified. According to tandem MS data, the site of modification was the thiol function.

Incubations of plasma with 3-PBA glucuronide; isolation of albumin

To 0.5 ml of human plasma was added 5 μl of a solution of 3-PBA glucuronide (**3**) in various concentrations (end concentrations in plasma: 0.5 mM, 0.05 mM, 0.005 mM and 0 mM as a control). After incubation for 2h at 37ºC, 2 ml of buffer A (50 mM KH_2PO_4, pH 7.0) was added. The solutions were filtered through 0.45 μm acrodisc filters and albumin was isolated over a HiTrap Blue HP affinity column. This column was equilibrated with 10 ml A buffer, followed by application of the sample. The impurities were removed by flushing the column with 10 ml A buffer, followed by elution of the albumin with 3 ml B buffer (50 mM KH_2PO_4 + 1.5M KCl, pH 7.0). Between the samples the column was consecutively flushed with 5 ml B-buffer and 20 ml A-Buffer. Subsequently, the purified albumin samples were desalted over a PD-10 desalting column, which had been equilibrated with 25 ml NH_4HCO_3 solution (50mM). After applying the samples, the columns were eluted with 3 ml NH_4HCO_3. These albumin solutions were used for enzymatic digestion.

Pronase digestion

To 750 μl of the albumin solution was added 100 μl of pronase solution (10 mg/ml 50 mM NH_4HCO_3). This mixture was incubated for 2 h at 37ºC and filtered over a 10kD filter before analysis with LC-MS(/MS).

Trypsin digestion

An aliquot (0.5 ml) of the albumin solution was lyophilized and dissolved in buffer (0.3 ml; 6 M guanidine.HCl, 100 mM Tris, 1 mM EDTA, pH 8.3). To this solution was added

dithiothreitol (5 mg) and the mixture was incubated for 40 min at 55ºC. Next, monoiodoacetic acid (10 mg) was added and the mixture was incubated for another 30 min at 40ºC. The solutions were transferred into a Slide-a-lyzer cassette and dialyzed overnight against 50 mM NH_4HCO_3. To the dialyzed albumin solution ($\pm$ 3 mg albumin) was added trypsin solution (30 µl; 1 µg/µl in 50 mM NH_4HCO_3). This mixture was incubated for 4 h and filtered over a 10kD filter before analysis with LC-tandem MS.

Incubations of plasma with 3-PBA glucuronide and Cl_2CA glucuronide; isolation of albumin

To 0.5 ml of human plasma was added 5 µl of a solution of 3-PBA glucuronide in various concentrations (end concentrations in plasma: 0.5 mM, 0.05 mM, 0.005 mM and 0 mM as a control). After incubation for 2h at 37ºC, 2 ml of buffer A (50 mM KH_2PO_4, pH 7.0) was added. The solutions were filtered through 0.45 µm acrodisc filters and albumin was isolated over a HiTrap Blue HP affinity column. This column was equilibrated with 10 ml A buffer, followed by application of the sample. The impurities were removed by flushing the column with 10 ml A buffer, followed by elution of the albumin with 3 ml B buffer (50 mM KH_2PO_4 + 1.5M KCl, pH 7.0). Between the samples the column was consecutively flushed with 5 ml B-buffer and 20 ml A-Buffer. Subsequently, the purified albumin samples were desalted over a PD-10 desalting column, which had been equilibrated with 25 ml NH_4HCO_3 solution (50mM). After applying the samples, the columns were eluted with 3 ml NH_4HCO_3. These albumin solutions were used for enzymatic digestion.

Pronase digestion

To 750 µl of the albumin solution was added 100 µl of pronase solution (10 mg/ml 50 mM NH_4HCO_3). This mixture was incubated for 2 h at 37ºC and filtered over a 10kD filter before analysis with LC-MS(/MS).

Trypsin digestion

An aliquot (0.5 ml) of the albumin solution was lyophilized and dissolved in buffer (0.3 ml; 6 M guanidine.HCl, 100 mM Tris, 1 mM EDTA, pH 8.3). To this solution was added dithiothreitol (5 mg) and the mixture was incubated for 40 min at 55ºC. Next, monoiodoacetic acid (10 mg) was added and the mixture was incubated for another 30 min at 40ºC. The solutions were transferred into a Slide-a-lyzer cassette and dialyzed overnight against 50 mM NH_4HCO_3. To the dialyzed albumin solution ($\pm$ 3 mg albumin) was added trypsin solution (30 µl; 1 µg/µl in 50 mM NH_4HCO_3). This mixture was incubated for 4 h and filtered over a 10kD filter before analysis with LC-tandem MS.

Synthesis of lysine adducts of 3-PBA and Cl_2CA

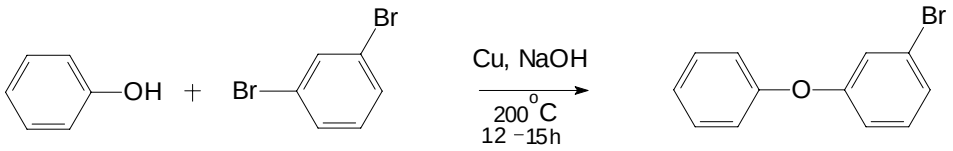
The synthesis was carried out on a PHB-S-TG resin (Rapp Polymere; 0.24 mmol/g resin) containing immobilized Boc-Lys(Fmoc) on a 36 μmol scale. The resin (150 mg) was swollen in DMF (3 ml) in a peptide synthesis tube for 45 min. Subsequently, the resin was treated with piperidine/DMF (8/2, v/v), 4 × 4 min, and washed with DMF (5x 2 ml). To the resin were added 3-PBA or Cl_2CA (10 eq, in 1 ml of DMF), PyBOP (10 eq, in 1 ml of DMF), HOBT (10 eq, in 1 ml of DMF) and DIPEA (20 eq). The synthesis tube was gently shaken every 15 min. After 2.5 h the resin was washed with DMF (5x 2 ml), dichloromethane (4x 0.2 ml), dichloromethane/diethyl ether (50/50, v/v; 4 x 0.2 ml) and diethylether (4x 0.2 ml), consecutively. The resin was air dried overnight. Deprotection and cleavage from the resin was carried out as follows. To the resin was added TFA/TIS (95/5, v/v; 6x 0.6 ml with 5 min interval). After the last addition, the liquid was pushed through the tube with a plunger. The cleaved lysine derivative was left for 2 h (after first addition) in the TFA/TIS solution. The solution was concentrated under a stream of nitrogen. To the concentrated compound was added diethyl ether/pentane (1/1, v/v; 10 ml), upon which a precipitate was formed. The precipitate was isolated by means of centrifugation and was washed with diethyl ether/pentane (1/1, v/v; 10 ml). The solid material was air dried overnight, taken up in water (1 ml), and lyophilized. The compounds were analyzed by HPLC and LC-tandem MS and were considered to be pure enough (> 90%) for use as reference compounds.

Enzymatic synthesis of β-glucuronides of 3-PBA and Cl_2CA, followed by incubation with human plasma

First, experiments were carried out in order to demonstrate that the glucuronides of 3-PBA and Cl_2CA could be prepared in this way. Thus, to an Eppendorf tube containing 3-PBA (25.6 μg), a solution (250 μL) of UDP-acetylglucosamine (1.2 mM) and UDP glucuronic acid (6 mM) in Tris buffer (120 mM Tris; 1% DMSO; 6 mM $MgCl_2$; pH 7.4), a suspension of microsomes (20 mg/ml in sucrose solution; 50 μl) was added. The mixture was incubated for 4 h at 37 ˚C. Subsequently, a mixture of TFA/CH_3CN (4/96, v/v; 63 μl) was added in order to precipitate the proteins. The sample was centrifuged and the supernatant was analyzed by means of LC tandem MS, that demonstrated the presence of the desired glucuronide. In an analogous way, the β-glucuronide of Cl_2CA was prepared and its identity was confirmed by MS.

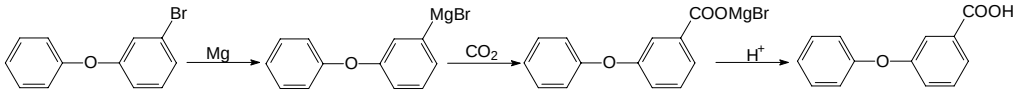
In case of exposure of human plasma to the glucuronides, the quenching step was omitted and the plasma (0.3 ml) was added directly to the glucuronidation mixture, after 4 h of incubation at 37 ºC. Albumin was isolated and digested with pronase as described above.

Synthesis of meta-bromodiphenyl ether (to be used for synthesis of [^{14}C] 3-PBA)



In a 50 ml 3-necked round bottom flask, equipped with a condenser, phenol (105 mmol) and NaOH (83 mmol) were stirred at 130-140 ºC (oil bath), until the NaOH had been dissolved. The reaction mixture was cooled to 100 ºC, and 8,4 mg copper powder was added. 1,3-dibromobenzene (20 ml, 0,1654 mol) was added dropwise. The temperature of the reaction mixture was increased to 200 ºC. The course of the reaction was followed by TLC. After 15 h, the reaction had gone to completion, and the reaction mixture was worked up by vacuum distillation. Further purification was performed by silica gel column chromatography (eluent: dichloromethane / hexane; gradient: 0-8% dichloromethane in hexane). Yield: 3 g (16%). Purity according to GC-MS: 97.7 %

Synthesis of 3-PBA (cold run)



The device represented in Figure 2 was used for the multi-step synthesis of 3-PBA; all reagents were present in the manifold prior to the beginning of the experiment.

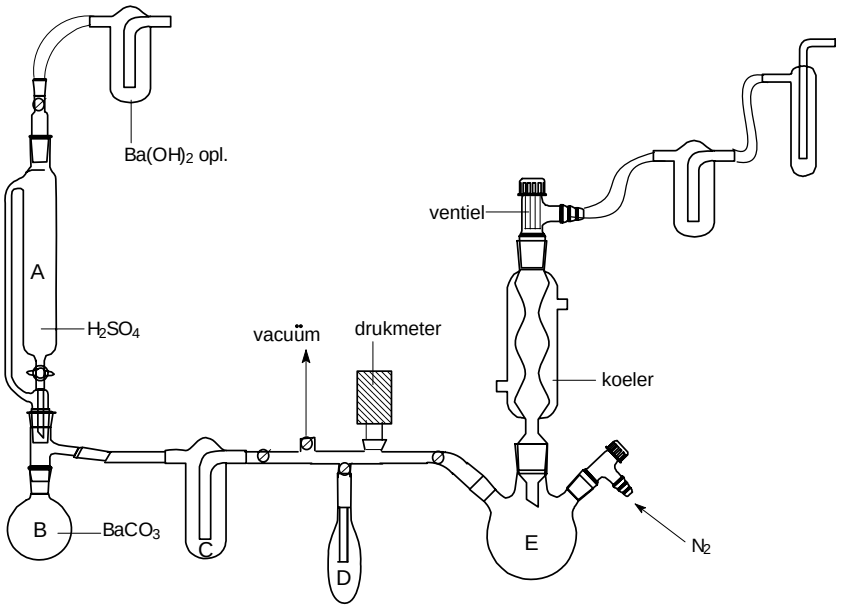


Figure 2. Manifold for preparation of [^{14}C] 3-PBA.

Preparation of the Grignard reagent

To flask E, containing magnesium (52 mg, 2 mmol) and a few crystals of iodine was added a solution of 3-bromodiphenylether (750 mg, 3 mmol) in diethyl ether (3 mL). The entire system was kept under nitrogen.

The preparation of the Grignard reagent was initiated by applying heat using a heat gun. When the reaction started, the mixture was refluxed gently until all of the magnesium had disappeared. The content of the flask was frozen by using a bath of liquid nitrogen and the entire system was evacuated using a vacuum pump.

Generation of CO_2

The following reaction occurred:

$BaCO_3$ (s) + H_2SO_4 (l) → $BaSO_4$ (aq) + CO_2 (g) + H_2O (l)

Vessel A was charged with sulfuric acid (8 mL) and the left part of the manifold was brought to vacuum. By using a heat gun, the sulfuric acid was heated, until no gas development was visible anymore. To flask B had already been added $BaCO_3$ powder (165 mg, 0.84 mmol). The left part of the manifold was evacuated again, while condenser C was cooled with solid CO_2/acetone and condenser D with liquid nitrogen. The connection between the vacuum pump and the manifold was closed and generation of CO_2 was started by dropping a few

drops of concentrated sulfuric acid onto the $BaCO_3$ powder. After the first violent development of CO_2, the remaining sulfuric acid was added in a few minutes and the remaining solution was stirred and heated with a heat gun. The generated CO_2 was dried by passing through condenser C and condensed to a white solid in condenser D. Finally, the valve between condenser C and D was closed.

Synthesis of 3-PBA

The connection with the pump was closed and the valve between condenser D and the right part of the manifold was opened. Flask E was warmed up in a bath of acetone/CO_2, while condenser D was allowed to warm up to room temperature allowing the solid CO_2 to sublimate and condense in flask E. Reaction started between CO_2 and the Grignard and the reaction was stirred for another 15 min at room temperature.

Next, nitrogen was flushed through the system and water (1.5 mL) was added to the content of flask E, followed by concentrated HCl (12 M, 1.5 mL). Diethyl ether (5 mL) was added and the mixture was transferred to a separation funnel. The organic layer was extracted with water (2 x 5 mL) and evaporated to a small volume.

A solution of NaOH (0.6 g/10 mL water) was added and the solution was extracted with CH_2Cl_2 (3 x 5 mL). The water layer was acidified using concentrated HCl (3 mL) and extracted again with CH_2Cl_2 (4 x 10 mL). The combined organic layer was dried ($MgSO_4$) and concentrated. Yield 186 mg (0.87 mmol, 43 %).

Synthesis of [^{14}C] 3-PBA

For the preparation of [^{14}C] 3-PBA, almost the same protocol as described above was used. To flask B was added [^{14}C]$BaCO_3$ (165 mg, 5 mCi, 0.84 mmol; sp. act 6 mCi/mmol); we used a larger excess 3-bromodiphenylether (4 mmol, 1 gram) and magnesium (3 mmol, 72 mg) than during the cold run, in order to give a maximum conversion of [^{14}C] CO_2. After stirring the Grignard reaction mixture for 15 min, acidifying took place with HCl (4 mL, 6 M), followed by a wash step with water (3 x 3 mL). The diethyl ether layer was evaporated to dryness and dissolved in CH_2Cl_2 (10 mL) and extracted with a solution of NaOH (600 mg in water (10 mL)). The organic layer was discarded, while the alkaline water layer was extracted three more times with CH_2Cl_2 (3 x 5 mL). The water layer was acidified with HCl (12 M, 4 mL) and extracted with CH_2Cl_2 (4 x 5 mL). The organic layer was dried (MgSO4). Total yield was 0.5 mCi (10 % overall). The obtained product was purified by HPLC in order to remove a minor contamination (2-PBA; 3%). Analysis with LC/MS of the purified portion confirmed the correct mass and mass spectrum after comparison with commercial “cold” 3-PBA.

Preparation of [^{14}C] 3-PBA-glucuronide

[^{14}C] 3-PBA (43 microgram, 0.2 micromol, sp. act. 6 mCi/mmol) was dissolved in buffer (120 mM Tris, 1% DMSO, 6 mM $MgCl_2$, 1.2 mM UDP acetylglucosamine, 6 mM UDP glucuronic acid; 425 microliter). To this solution, a suspension of human liver microsomes (75 microliter) was added. The suspension was incubated for 4 h at 37 ºC. Reversed phase HPLC analysis showed a conversion of ca 35% to a more polar compound. The formed 3-PBA glucuronide was purified by semi-preparative HPLC. HPLC analysis of a part of the purified reaction mixture showed a single radioactive peak, having the expected retention time (comparison with synthetic reference compound).

HPLC conditions:

Buffer A: 5% CH_3CN/H_2O, 0.1 % TFA

Buffer B: 80 % CH_3CN, 0.1 % TFA

Column: Alltech C18 (250 mm * 4.6 mm, 5 micron)

HPLC system: Perkin Elmer 200 series

Detector: Perkin Elmer UV detector, 200 series

Radiomatic detector: Perkin Elmer Radiomatic flow scintillation analyzer

Gradient:

0-1 min 0% B

1-4 min 0 to 50 % B

4-19 min: 50 to 75 %B

19-20 min: 75 to 100% B

20-23 min: 100% B

23-24 min: 100 to 0 % B

24-27 min: 0 % B

MS analysis confirmed the structure of the new formed compound as [^{14}C] 3-PBA-glucuronide (*m/z* 389 for native compound, 391 for ^{14}C-labelled compound). Appropriate fractions were combined, lyophilized and incubated with human plasma (20 micromolar end concentration of glucuronide) for 2 h at 37 ºC.

Preparation of 3-PBA-[^{14}C] glucuronide

To a lyophilized portion 3-PBA (43 microgram) was added a solution of [^{14}C] UDP-glucuronic acid (250 microliter, ethanol/water 7/3 v/v, 180 mCi/mmol, 5 microCurie). The solution was evaporated to dryness in a vacuum centrifuge. Buffer (120 mM Tris, 1% DMSO, 6 mM $MgCl_2$, 1.2 mM UDP acetylglucosamine; 425 microliter) and human liver microsomes (75 microliter) were added. The suspension was incubated for 4 h at 37 ºC. HPLC analysis showed a conversion of approximately 20 % to a compound with a similar retention time as 3-

PBA-glucuronide. This product was isolated using a Seppak C_{18} (classic) cartridge, because of the large difference in retention time of labeled UDP-glucuronic acid and 3-PBA glucuronide. The cartridge was conditioned with HPLC buffer B (3 mL) followed by HPLC buffer A (3 mL). The reaction mixture was diluted with water (1 mL) and applied to the Seppak cartridge, followed by 2 wash steps with buffer A (2 x 3 mL). Next, the 3-PBA-[^{14}C]glucuronide was eluted using buffer B (2 mL). The appropriate fraction was lyophilized and used for incubations with plasma.

Incubation of [^{14}C] 3-PBA glucuronide with human plasma, sample work-up and quantitation of binding

Incubation of [^{14}C] labelled 3-PBA glucuronides with human plasma (20 micromolar end concentration of glucuronide) was performed for 2 h at 37 ºC. It was estimated that the protein content in plasma was 57 mg/mL. After the incubations of purified 3-PBA-glucuronides with plasma, it was attempted to separate bound from unbound 3-PBA-glucuronides. First, molecular weight cut-off filters (0.5 mL) with a cut-off of 3 kD were used, which were washed with a solution of 20 % CH_3CN/PBS. This method was very time consuming and the use of acetonitril probably caused clogging of protein material. Use of similar filters with a cut-off of 10 kD was more successful. Small plasma samples (0.2 mL) could be washed very frequently with PBS (18 x 1 mL) at 4000g. The PD-10 column was equilibrated with buffer (50 mM NH_4HCO_3, 25 mL). The sample was diluted to a volume of 2.5 mL with the same buffer and applied to the column. The column was eluted using 50 mM NH_4HCO_3 in 1 mL portions. From each fraction, 0.5 mL was used for liquid scintillation countings. Protein contents of PD-10 fractions were determined using a modified Lowry protocol (RC DC Protein Assay, BioRad). Further quantitation of binding was studied more careful, e.g. by extensively washing the plasma retentates on a 10 kD molecular weight cut-off filter with 6 M guanidine buffer, followed by a final PD-10 column of the retentate (in PBS), in order to get rid of all non-covalently bound material. Thus, plasma (0.5 mL) was incubated with purified [^{14}C]3-PBA-glucuronide at levels of 20 and 40 micromolar for 2 h at 37 ºC. The plasma samples were diluted with guanidine buffer (6 M) to an end volume of 4 mL. Then, part (3 mL) was filtrated over a 10 kD MWCO filter and the retentate was washed with guanidine.HCl (6M, 4 x 4 mL). The remaining part of the diluted plasma sample (0.75 mL) was washed with urea (8 M, 4 x 4 mL) for comparison reasons. After the washings, the retentates were analyzed for remaining radioactivity.

Protein extraction from liver

Monkey liver was homogenized with a Potter homogenizer at 800-1000 rpm in 10 volumes of ice cold 50 mM Tris buffer, pH 8, supplemented with 0.32 M sucrose and a 1/10 v/v of

protease inhibitor cocktail solution, obtained by dissolving one buffer protease inhibitor cocktail tablet in 1.5 mL of deionized water. The suspension was centrifuged at 1000*g* for 10 min at 4 °C, and the supernatant was centrifuged at 17000*g* for 55 min at 4 °C. Then, the supernatant was centrifuged at 100000*g* for 60 min at 4 °C.

The thus obtained supernatant was stored in aliquots at -70 °C. Protein concentrations were determined with the RC/DC protein assay.

Exposure of plasma or liver homogenate to 3-PBA glucuronide

Plasma or liver homogenate (0.09 mL) was incubated with purified [^{14}C]-3-PBA-glucuronide (56 mCi/mmol) at levels of 0.1, 0.5 or 1 mM. The purified [^{14}C]-3-PBA-glucuronide was lyophilized and dissolved in DMSO/water (10% v/v, 10 microliter). Next, plasma or liver homogenate was added and the mixture was incubated for at least two h at 37 ºC. After incubation, the samples were stored at -20ºC.

Electrophoresis and visualization

For electrophoresis, two parallel gels were run, each loaded with 200 ug protein from liver or plasma both solubilized in 50 uL sample buffer containing 0.5 M Tris, pH 6.8 , 33% (v/v) 10% SDS, 17% (v/v) glycerol, 8% (v/v) β-mercaptoethanol, 8% (v/v) 0.1% Bromophenol blue. Proteins were separated on lab-cast 10 % polyacrylamide gels with a 4% stacking gel using a Hoefer 600 electrophoresis unit (Hoefer, Holliston, MA, USA). Gels were run in electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 15mA/gel for 15 min followed by 30mA/gel, at 20 °C until the bromophenol blue had migrated to the bottom of the gel. After gel electrophoresis one gel was stained with Coomassie blue following protocol (BioRad) and the other gel was fixed with methanol-acetic acid-water 25:10:65 (v/v/v).

For autoradiography both gels were dried on filter paper, using a gel drier (BioRad). Both dried gel was exposed to [^{14}C] sensitive imaging plates (BioRad). Afterwards, the imaging plates were enclosed in exposure cassettes (BioRad) and exposed at room temperature for 14 days. At the end of the exposure time, the gels were removed from the imaging plate and the plates were then scanned using the Typhoon 9400 storage phosphor screen (resolution 200 µm) (GeHealthcare, Upsulla, Sweden).

In vivo exposure of guinea pigs to permethrin

A guinea pig was exposed to permethrine in vivo (20 mg/kg, s.c.). Blood samples (ca 0.25 mL) were taken at several days after injection and separated into red blood cells and plasma. A first blood sample was taken before the injection of permethrin and this was considered as the blank.

Of each plasma sample, 0.125 mL was mixed with a solution of pronase in buffer (10 mg/ml, 50 mM NH_4HCO_3, 0.1 mL) and incubated for 2 h at 37 °C. Next, the mixture was centrifuged for 30 min in an Eppendorf centrifuge using a 3 kD filter with a capacity of 0.5 mL. The filters were rinsed first with water (0.5 mL). The filtrates were analyzed using LC/MS.

Analysis and work-up of plasma samples from individuals exposed to permethrin

Plasma samples (0.125 mL) were incubated with a solution of pronase (0.1 mL, 10 mg/mL) in buffer (50 mM NH_4HCO_3) for two h at 37º. Next, the digested samples were filtrated via a prewashed (0.2 mL water) 3 kD filter using an Eppendorf centrifuge and analyzed with LC/MS for 3-PBA adducts with lysine.

IV RESULTS AND DISCUSSION

Synthesis of 3-PBA-glucuronide

3-PBA-glucuronide was synthesized according to two slightly different procedures (see Figure 3). The yields of the first synthesis method (Kenny et al., 2004) was very low. In this case, a mixture of α and β-anomers resulted, having the correct mass that could be separated after deprotection, i.e., removal of the allyl function The desired β-anomer could be obtained in mg amount and could be fully characterized. A more recent method (Perry et al., 2005) was applied for synthesis of this compound. In this case, the yield was much higher and only the desired β-anomer resulted.

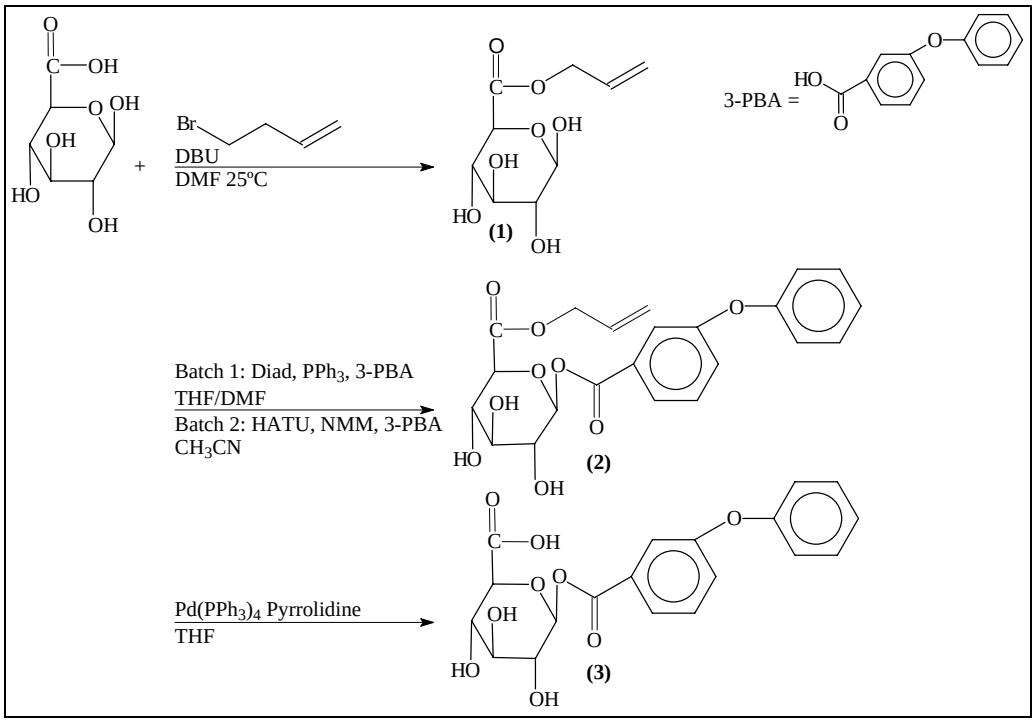


Figure 3. Synthesis of 3-PBA glucuronide (**3**).

Deprotection proceeded rather smoothly; the end product was purified by means of silica gel column chromatography and semi-preparative reversed phase HPLC. See Figure 4 for LC-MS and LC-tandem MS analysis; see figures 1A and 2A in appendix for NMR spectra.

Overall, the synthesis of this compound is a difficult task, which is inter alia caused by its instability. Upon storage in solution, a rapid isomerization could be observed with HPLC-UV analysis and LC-MS analysis, indicating the intrinsic lability of the compound.

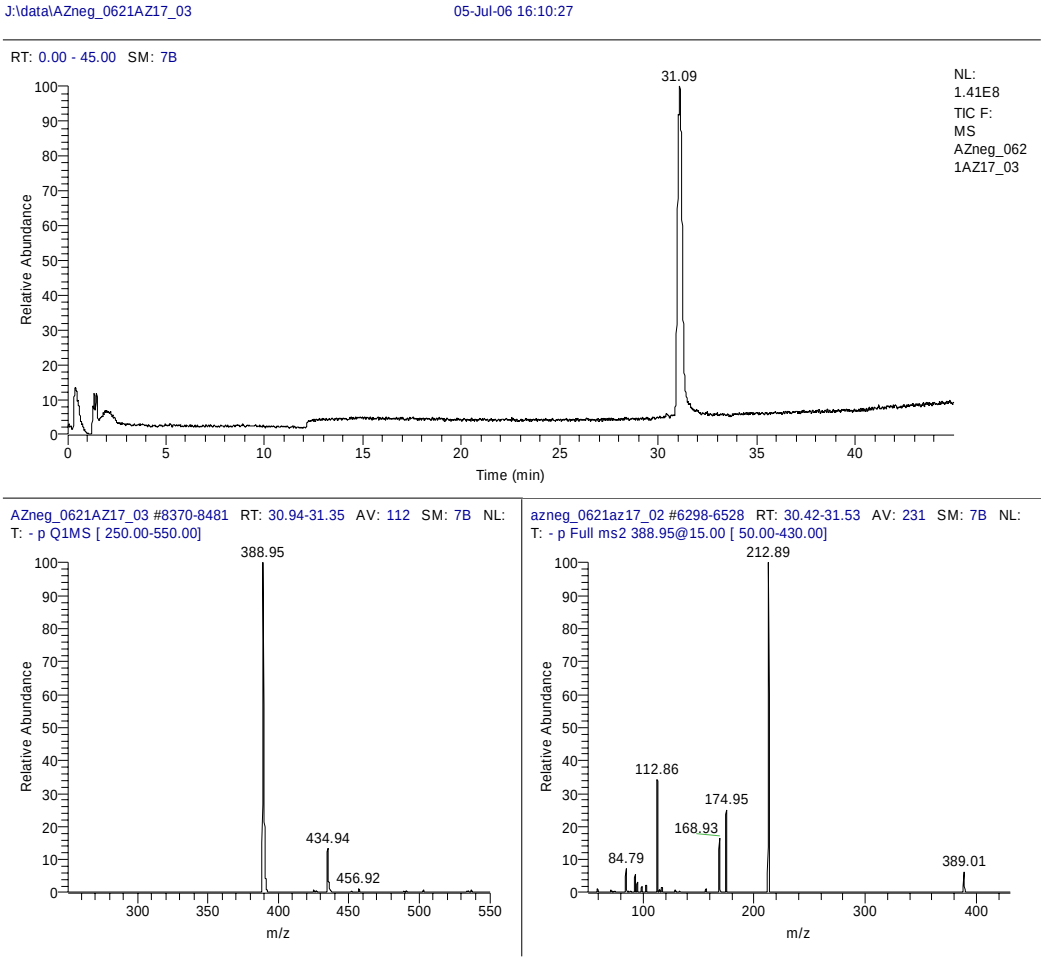


Figure 4. LC-MS and LC tandem MS spectra of 3-PBA β-glucuronide. The upper panel represents the total ion current (ES-). The lower, left panel is the corresponding mass spectrum; 389 $[M-H]^-$; 434.9 $[M + HCOO]^-$. The lower, right panel represents the tandem MS spectrum; 212.9 [3-PBA]-, 174.9 [389 – 3-PBA]$^-$, 168.9 [3-PBA – CO_2]$^-$, 112,8 [174.9 – H_2O – CO_2].

Synthesis of Cl_2CA-glucuronide

The synthesis of this acyl glucuronide (see Figure 5) was hampered by the fact that although we used a method (Perrie et al., 2005) that should result in exclusively the β-anomer (as was indeed the case for 3-PBA glucuronide), a significant amount of α-anomer resulted. Unfortunately, these isomers could not be separated, neither in the protected stage, nor in the deprotected stage. Another complicating factor in the synthesis was the fact that the end product was not longer UV-positive; therefore, following of the course of the reaction was problematic. Yet an another complicating factor was the fact that Cl_2CA consists of a mixture

of E and Z isomers. We decided to perform the preliminary binding experiments with the enantiomeric mixture. For mass spectrometric data: see Figure 6.

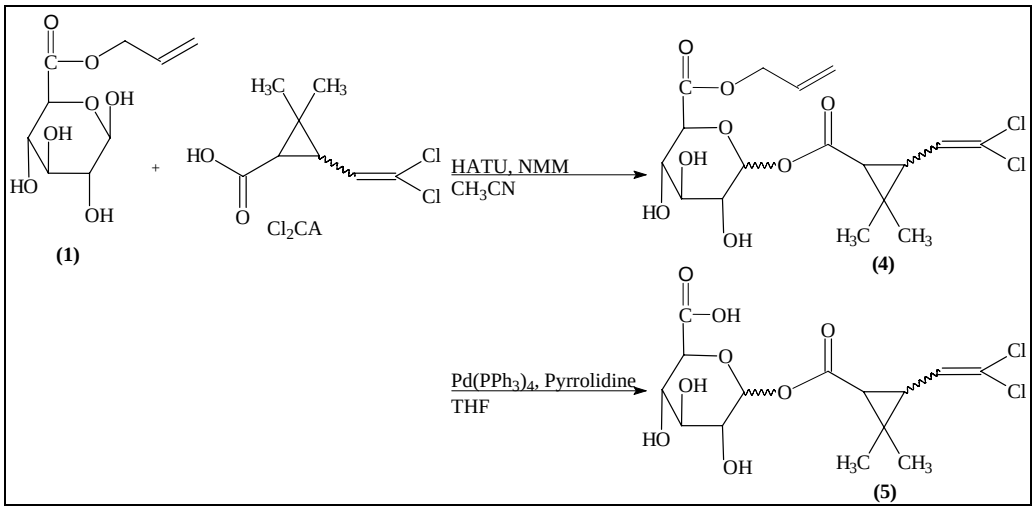


Figure 5. Synthesis of Cl_2CA-glucuronide (**5**).

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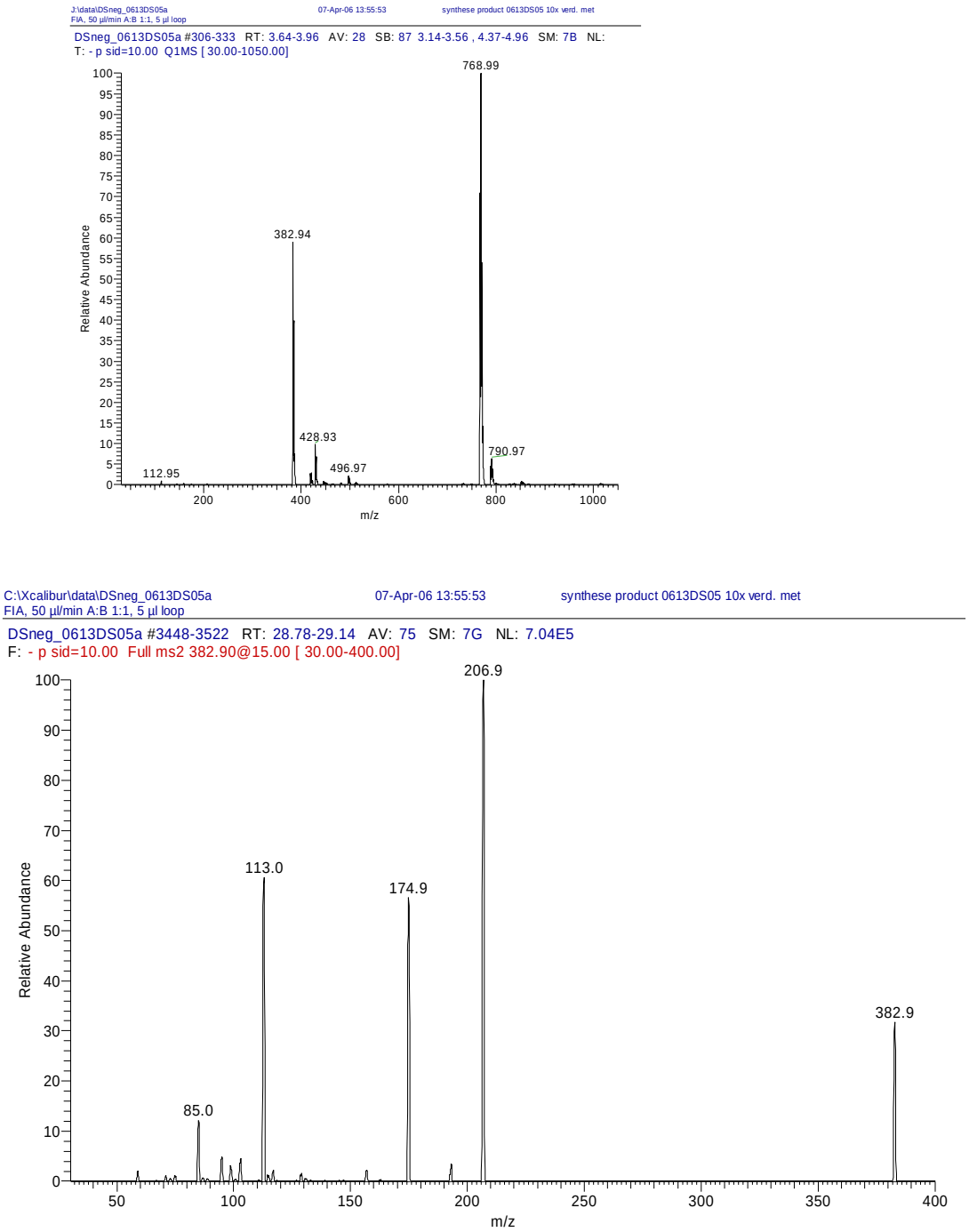

Figure 6. Mass spectrum (upper trace) of Cl_2CA glucuronide; 382.9 $[M-H]^-$, 428.9 [M+HCOO-]-, 768.9 [2M-H]-. Tandem MS spectrum of Cl_2CA glucuronide (lower trace): 206.9 $[Cl_2CA]$-, 174.9 $[382.9 – Cl_2CA]$-, 113.0 $[174.9 – H_2O – CO_2]$.

Adduct formation 3-PBA-glucuronide and Cl_2CA-glucuronide with model compounds

Introduction

Conjugation to glucuronic acid ("glucuronidation") by UDP-glucuronosyltransferase-mediated transfer of a glucuronyl moiety of UDP-glucuronic acid to a nucleophilic site of a xenobiotic is one of the major Phase II detoxicification reactions. It renders the xenobiotic more polar which facilitates its excretion. This reaction takes place predominantly in the liver. In case of glucuronidation of a carboxylic acid, potentially reactive electrophilic acyl glucuronides result that can react with nucleophilic residues within the organism.

Two mechanisms of adduct formation by O-acyl glucuronides can be distinguished. According to the *transacylation* mechanism (Figure 7), nucleophilic sites in the proteins are acylated by the O-acyl glucuronide and consequently modified with the acyl moiety derived from the "original" metabolite. E.g., in case of the glucuronide of 3-PBA this should be a 3-phenoxybenzoyl moiety (see Figure 8 for the chemical structure of an expected adduct). According to the *glycation* mechanism, an initial internal acyl migration occurs, followed by reaction with amino groups of the protein, leading to so-called Schiff base adducts (Grubb et al., 1993; Smith et al., 1990), which may eventually undergo a (slow) Amadori rearrangement (see Figure 9). However, the latter rearrangement has not been experimentally confirmed. In case of the glucuronide of 3-PBA and Cl_2CA the expected Schiff base adduct will have the chemical structure as shown in Figure 10.

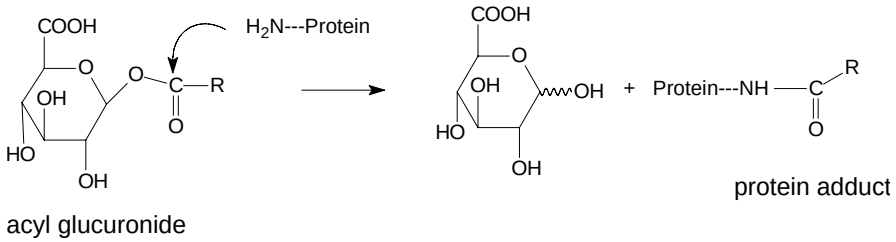

Figure 7. Adduct formation of acyl glucuronides with proteins by means of the transacylation mechanism.

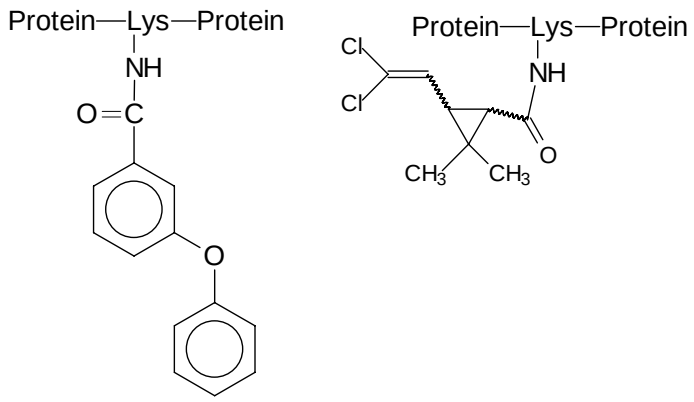

Figure 8. Schematic structure of a presumed lysine-adduct of permethrin-derived O-acyl glucuronides, according to the transacylation mechanism

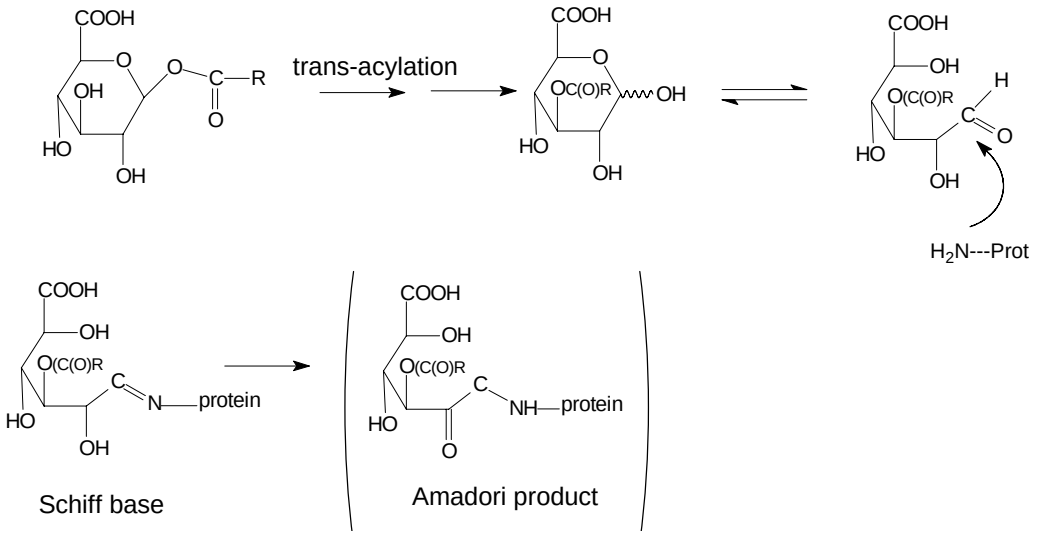

Figure 9. Adduct formation by acyl glucuronides via the glycation mechanism.

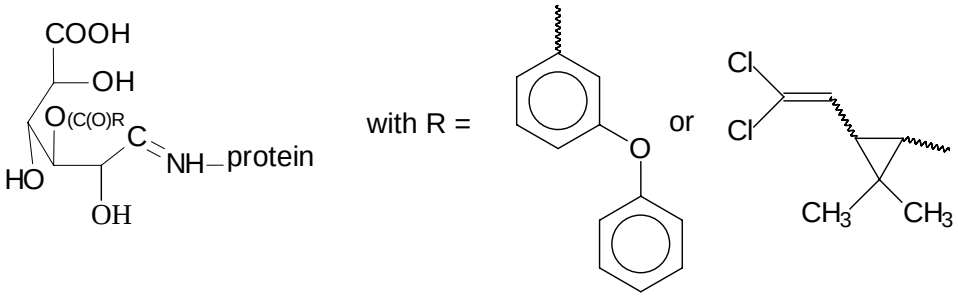

Figure 10. Identity of presumed adduct of permethrin-derived O-acyl glucuronide, according to the glycation mechanism.

In general, the acyl glucuronides are stable enough to enter the blood stream (see, Sallustio et al, 2000).

The most likely candidate for protein adduct formation by acyl glucuronides is human serum albumin (HSA), which is a rather abundant protein in the plasma (see, e.g., Presle et al., 1996; Qiu et al., 1998). It has been demonstrated that the lysine 195 and 199 residues in the hydrophobic pocket of subdomain IIA of HSA are preferentially modified by various acyl glucuronides (Ding et al., 1993, 1995; Zia-Amirhosseini et al., 1995). The most extensive investigations were performed on the reactivity of the glucuronide of the NSAID tolmetin with human serum albumin, with identification of the binding sites by means of tandem mass spectrometry (Ding et al, 1995). Lysine 199 reacted not only via the Schiff base mechanism , but also by nucleophilic displacement, as did lysine 541. However, for lysine 199 the Schiff base formation predominated.

Adducts to lysine residues are probably rather stable in vivo. Interestingly, we have recently shown that these particular lysine residues are also highly reactive towards the acylating agent phosgene (Noort et al., 2000). In this case an intramolecular adduct was formed, in which the lysine 195 and 199 residues were bridged intramolecularly by an urea-type chemical bond (with the carbonyl moiety derived from phosgene). It has also been demonstrated that these particular amino acid residues are modified by penicillin (Yvon et al., 1989), and that the resulting adduct is involved in allergic reactions. On the basis of previous research (Noort et al., 1999), it is likely that the cysteine-34 residue will react readily with the activated glucuronides, but whether the resulting thioester adduct is stable enough to accumulate in the organism, is not very plausible. However, Grillo et al (2003; see also Grillo and Hua, 2003 for corresponding adducts of NSAID zomepirac) found that the S-glutathione adduct of the

NSAID diclofenac was excreted from the bile of rats after administration of diclofenac glucuronide (see also Li et al, 2002).

Protein binding of glucuronides of benzoic acids that are structurally related to 3-PBA has been reported (see, *e.g.*, Akira et al., 2002). In more general terms, it appears that the degree of covalent binding to proteins of acidic drugs in man correlates well with the chemical reactivity of the glucuronides of these drugs (Benet et al, 1993). We envisaged that adduct formation with proteins of glucuronide derivatives of carboxylic acic metabolites of permethrin is probable and will provide a useful biomarker to assess cumulative exposure to this pyrethroid. We first selected some model compounds to explore whether the obtained acyl glucuronide are indeed electrophilic compounds.

Adducts of 3-PBA glucuronide

Several adducts resulted upon incubation of 3-PBA-glucuronide with glutathione, Z-Lys and the model peptides ASSAKQR and LKZASLQK, with Z = S-carboxymethylcysteine. The peptides have been derived from human serum albumin; the lysine (K) residues have been reported to be reactive towards acylating reagents (e.g., Noort et al., 2000) and also towards glucuronides (e.g., Ding et al, 1995). Characteristic for all incubations with 3-PBA-glucuronide is that the resulting adducts either show addition of 196 amu (-H + 3-phenoxybenzoic acid –OH) or of 372 amu (-H + 3-PBA glucuronide $–H_2O$), resulting from direct acylation or by reaction through the glycation mechanism.

In case of glutathione the binding site is the SH group, as could be assessed by the MS-MS spectrum of the adduct; adduct formation had occurred through the transacylation mechanim. See Figure 11 for the chemical structure of the glutathione 3-PBA adduct and Figure 12 for the MS-MS spectrum. There was also some evidence that an adduct had been formed through the glycation mechanism. Although the expected mass of such an adduct was detected, we could entirely solve the obtained tandem MS spectrum.

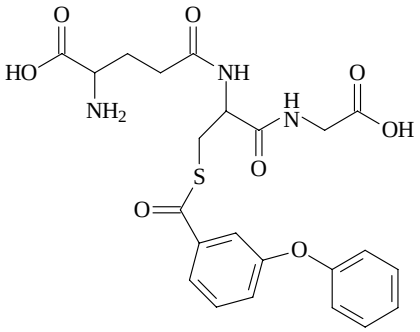

Figure 11. Chemical structure of glutathione 3-PBA glucuronide adduct.

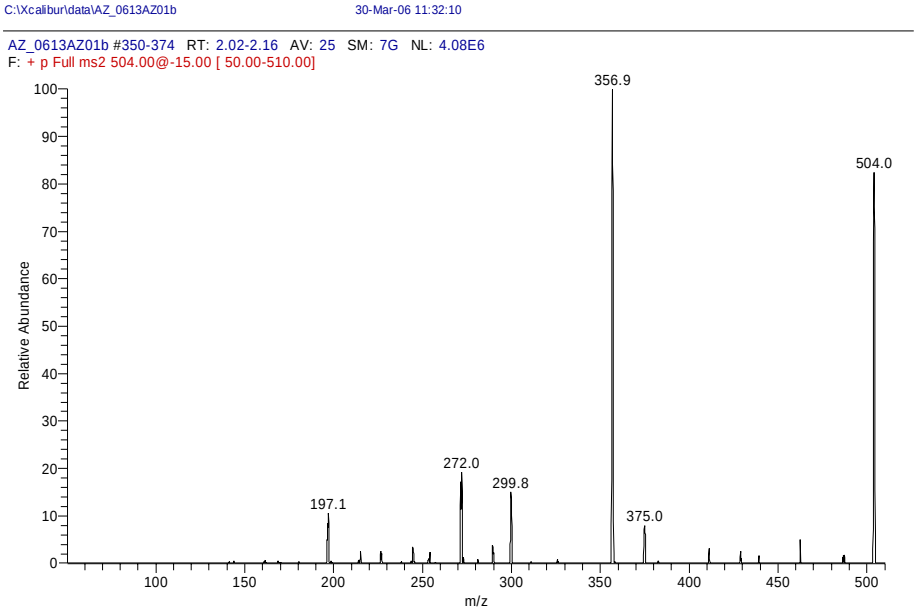

Figure 12. Tandem ES(+) MS spectrum of 3-PBA glucuronide adduct to glutathione. Typical fragments for 504.0 [M + H]+: 375.0 [y2"], 356.9 [y2"-H_2O], 299.8 [C(O)-NH-C=C-S-C(O)-3-phenoxyphenyl]+, 272.0 [H_3N-C=C-S-C(O)-3-phenoxyphenyl]+, 197.1 [C(O)-3-phenoxyphenyl]+

In case of Z-Lys-OH the binding site is the NH_2 group of lysine; the only observed adduct with m/z 477 $[M+H]^+$ was the adduct that had resulted from transacylation.

In case of incubation of 3-PBA glucuronide with ASSAKQR, transacylation adducts could be detected that were either derived from modification of the free amino group at the N-terminus, or from modification from the ε-amino group in lysine (see Figure 13). Also, adducts resulting from the glycation reaction resulted (see Figure 14).

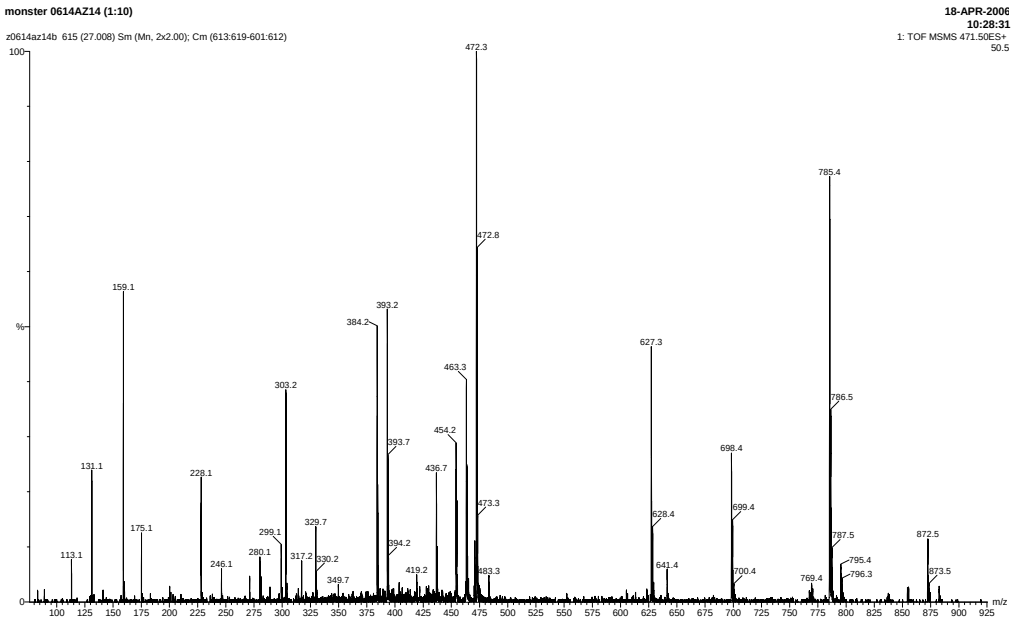

Figure 13. Tandem MS spectra of adduct of 3-PBA glucuronide with ASSAKQR, formed by the transacylation mechanism. Characteristic fragments: m/z 872.5 [y6" + 196], 785.4 [y5" + 196], 698.4 [y4" + 196], 627.3 [y3" + 196], 472.3 [MH_2^{2+}].

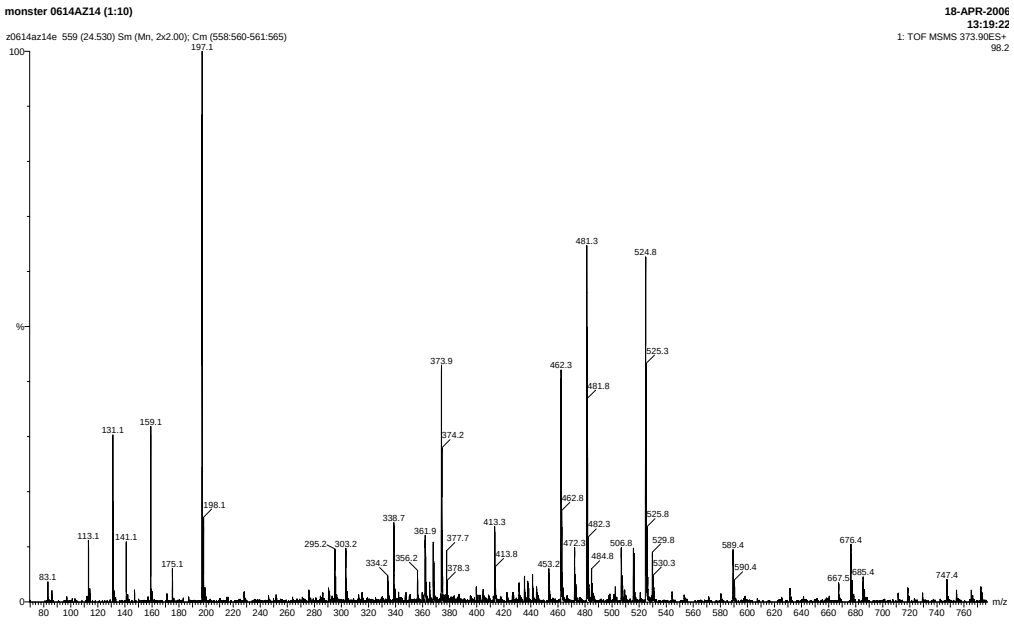

Figure 14. Tandem MS spectra of adduct of 3-PBA glucuronide with ASSAKQR, formed by the glycation mechanism. Characteristic fragments: 524.8 [y6" + 372.1], 481.3 [y5" + 372.1], 373.9 $[MH_3]^{3+}$, 197.1 $[C(O)\text{-}3\text{-}phenoxyphenyl]^{+}$.

In case of the model peptide LKZASLQK similar adducts were observed, formed by either the transacylation mechanism, or by the glycation mechanism.

In case of incubation of 3-PBA glucuronide with human plasma followed by pronase digestion of isolated albumin, the adduct derived from the tripeptide Cys-Pro-Phe containing the reactive Cys-34 residue, could not be observed. Probably this adduct is too unstable to survive the incubation with pronase; evidence for the lability of an S-acylated adduct was already obtained in our experiments with glutathione. After trypsin digestion of the albumin from the exposed plasma, no ASSAKQR adducts could be detected. However strong evidence was obtained for the formation of adducts, through either the transacylation or glycation mechanism, with LKZASLQK. In both cases, strong ion fragments were observed with m/z 197. Unfortunately, we could not yet identify unambiguously the site of modification.

Adducts of Cl_2CA-glucuronide

Incubation of glutathione with Cl_2CA-glucuronide resulted in the formation of a similar adduct as was observed for 3-PBA glucuronide, i.e., by modification at the thiol function; see Figure 15 for the ES+ tandem MS spectrum.

Adducts of Cl_2CA-glucuronide with Z-Lys-OH could not be detected; this might indicate that this particular type of acyl glucuronide is less reactive.

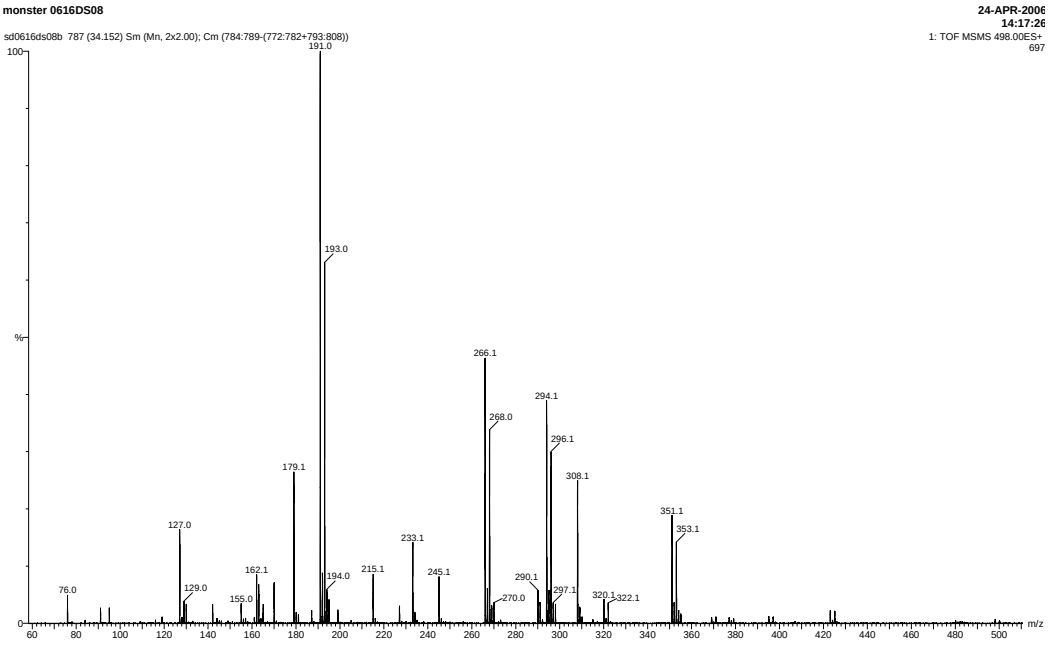

Figure 15. Tandem ES(+) MS spectrum of Cl_2CA-glucuronide to glutathione. Typical fragments for 498.0 [M + H]+: 351.1/353.1 [y2"-H_2O]; 294.1/296.1 [C(O)-NH-C=C-S-C(O)-dichlorovinyl-dimethylcylopropyl]+; 266.1/268.1 [H_3N-C=C-S-C(O)- dichlorovinyl-dimethylcylopropyl]+; 191.0/193.0, Cl_2CA fragment.

Incubations of 3-PBA and Cl_2CA β-glucuronides with human plasma, followed by enzymatic digestion

After trypsin digestion of the albumin from the 3-PBA-glucuronide exposed plasma, no ASSAKQR adducts could be detected. In the trypsin digest of plasma, incubated with 3-PBA-glucuronide, the fragment LK*ZASLQK with K* lysine modified according to the glycation mechanism was identified by LC-MS/MS. In case of exposure of plasma to Cl_2CA glucuronide, none of these peptides were found in the trypsin digest of albumin.

In addition to trypsin digestion, pronase digestion of albumin was explored. Advantage of pronase is that when modification of lysine is pronounced and quite random, the level of modified lysine in the digest will be much larger than the level of one specifically modified peptide fragment in the trypsin digest. First, the pronase digest of albumin was analyzed for the presence of modified (acylated or glycated) Cys*-Pro-Phe, after exposure of plasma to 3-PBA glucuronide or Cl_2CA glucuronide. Probably, the adduct (a thioester or thioether) is too unstable to survive the incubation with pronase.

Next, the pronase digests were analyzed for modified lysine. In case of exposure to 3-PBA glucuronide (0.5 mM – 5 mM) peaks with MH^+ 343.1 or MH^+ 519.1 were found in all samples, corresponding to a lysine derivative resulting from transacylation or glycation, respectively. The structures of the lysine adducts were confirmed with LC-MS-MS. The adduct resulting from glycation was more intense than the peak resulting from transacylation (see Figure 16 for ion chromatogram and Figure 17 for tandem MS spectrum. Similar results were found in the pronase digests of plasma incubated with Cl_2CA glucuronide (4.6 mM). Peaks with MH^+ 337.1 and 513 (broad peak) correspond to the modified lysine derivatives. In case of the transacylation adduct, LC-MS showed two peaks for the individual isomers (see Figure 18 and 19). Interestingly, for Cl_2CA glucuronide the adduct resulting from transacylation was the more intense adduct, which is in contrast to the results obtained for 3-PBA glucuronide.

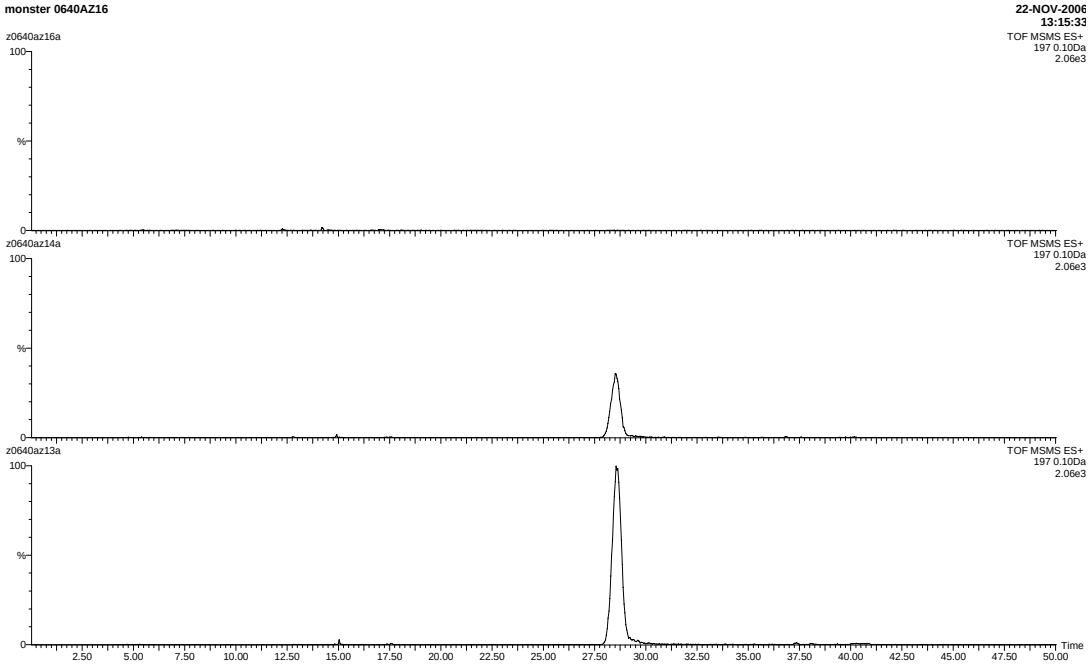

Figure 16. Ion chromatograms for 3-PBA-modified lysine (glycation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide. Upper trace, blank; middle trace, 1 mM exposure; 5 mM exposure.

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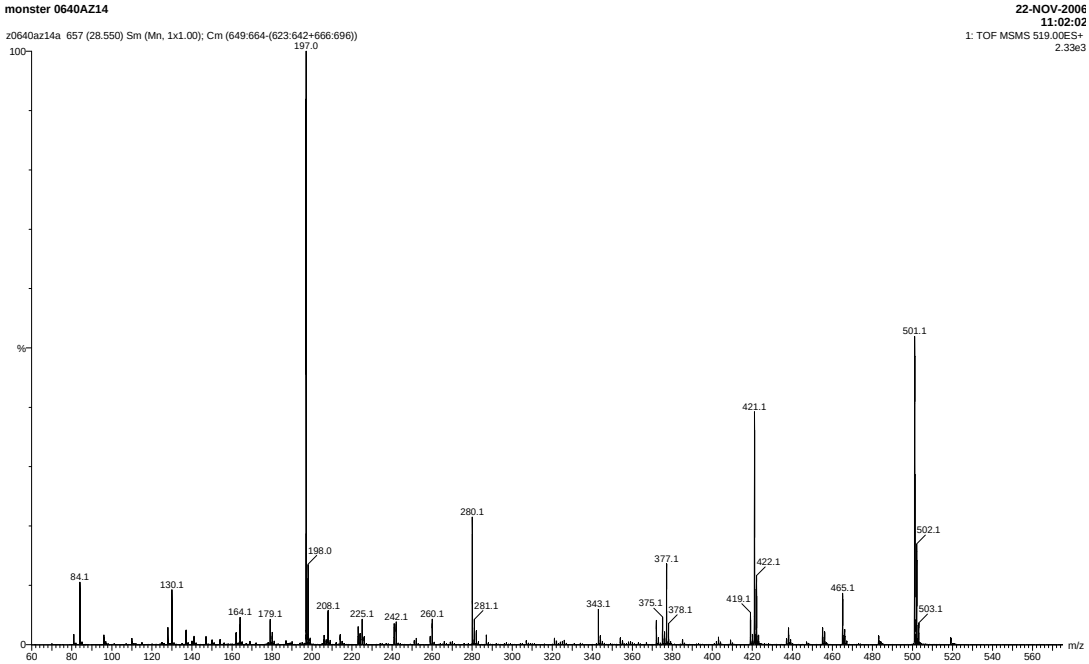

Figure 17. Tandem mass spectra of 3-PBA-lysine adduct (glycation) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide.

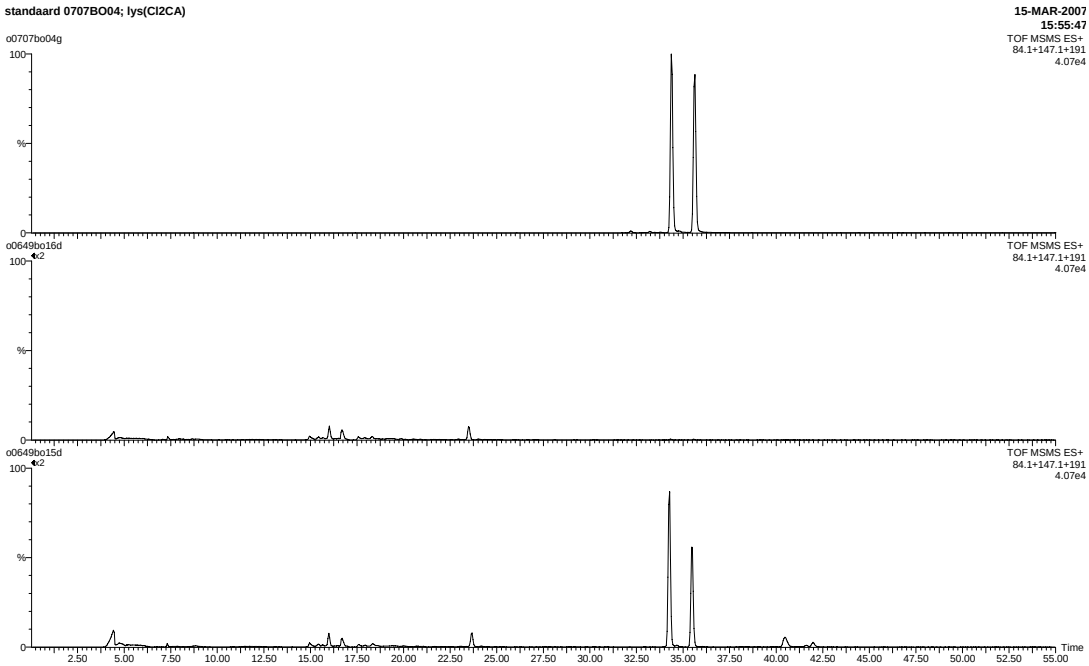

Figure 18. Ion chromatograms for Cl_2CA-modified lysine (transacylation adduct, both isomers) in a pronase digest of albumin isolated from human plasma that was incubated with synthetic Cl_2CA glucuronide. Upper trace, synthetic reference compounds; middle trace, blank plasma; exposed plasma.

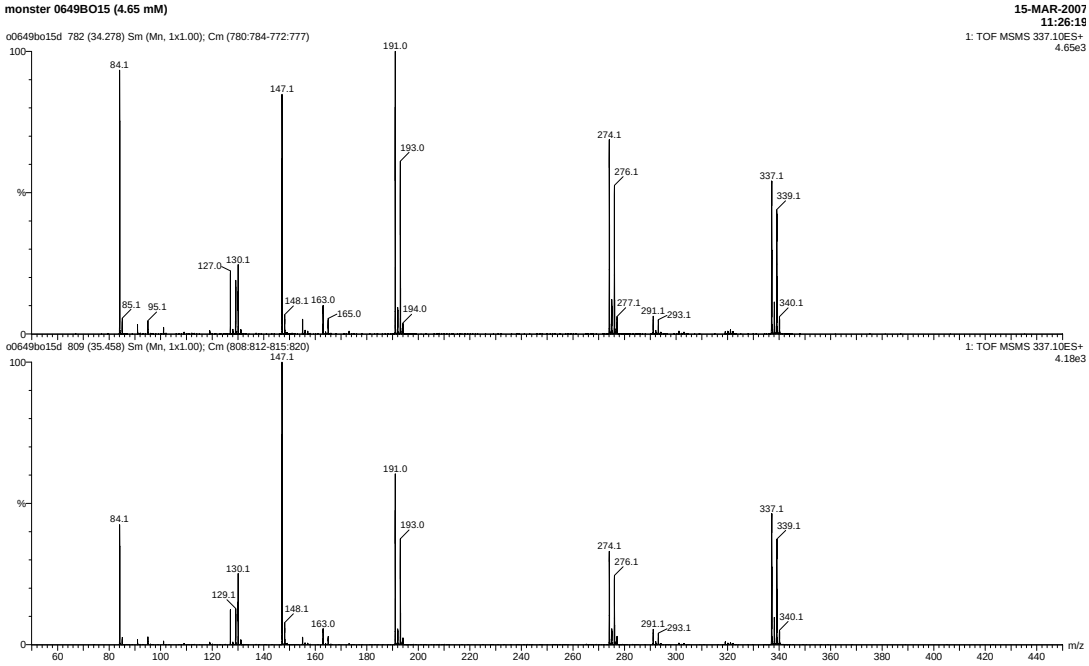

Figure 19. Tandem mass spectra of Cl_2CA-lysine adduct in a pronase digest of albumin isolated from human plasma that was incubated with Cl_2CA glucuronide. Upper trace: fast eluting isomer. Lower trace: slow eluting isomer (see Figure 18).

Care had to be taken with the results obtained with synthetic Cl_2CA glucuronide because a mixture of α/β-glucuronide was used. In order to ascertain that it was indeed the in vivo formed β-glucuronide was responsible for the adduct formation, we decided also to perform plasma incubations with β-glucuronide that had been prepared enzymatically, i.e., with liver microsomes and UDP-glucuronic acid. For this, the procedure published by Bolze et al (2002) was used. Plasma was exposed to the crude Cl_2CA-glucuronide, albumin was isolated and digested with pronase. The presence of the Cl_2CA-lysine adduct could be confirmed, although only one isomer of the adducts was visible, which is in contrast to the case when synthetic Cl_2CA-glucuronide was used (see Figure 20).

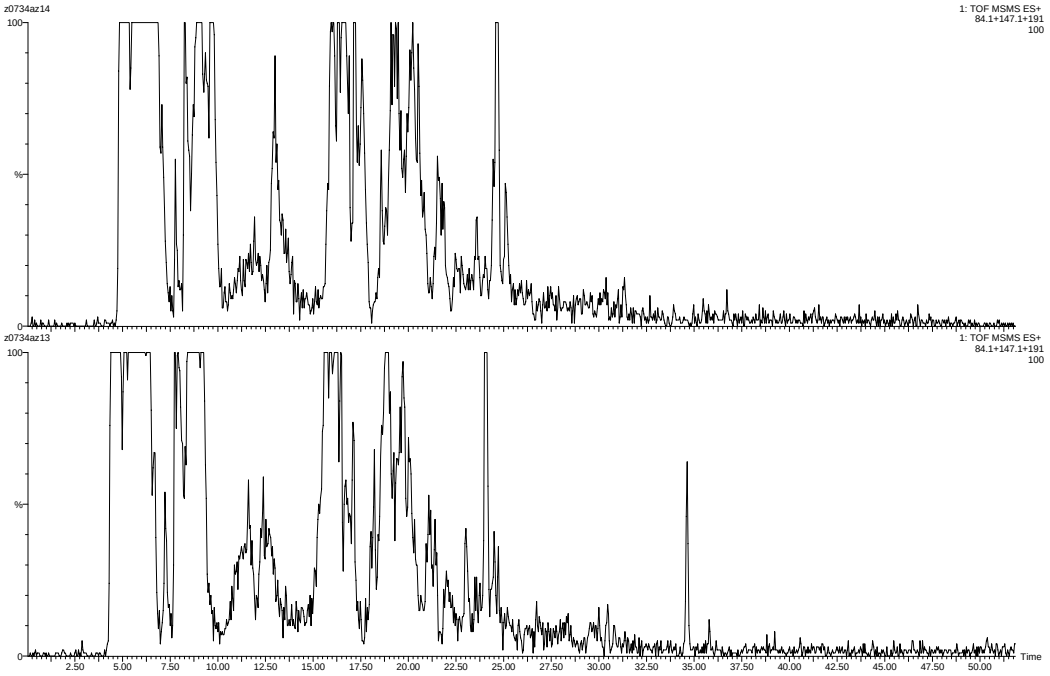

Figure 20. Ion chromatograms for Cl_2CA-modified lysine (transacylation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with Cl_2CA glucuronide, that was obtained through enzymatic synthesis. Upper trace, blank plasma; lower trace, exposed plasma. Peak at 34.5 min is peak of interest.

This might be explained by the fact that during biosynthesis of the Cl_2CA-glucuronide the various Cl_2CA isomers can display different reactivity, resulting in a different ratio of β-glucuronides and probably also in a different ratio of lysine adducts. Only the lysine adduct resulting from transacylation could be observed; this adduct was also the most pronounced adduct when synthetic Cl_2CA glucuronide was used. The β- glucuronide of 3-PBA was also prepared enzymatically. In this case, only the glycation adduct could be observed upon exposure of plasma to the crude glucuronide (see Figure 21).

For reference purposes, the lysine adducts of 3-PBA- and Cl_2CA glucuronide formed by transacylation, were prepared by using a solid phase peptide synthesis protocol, starting with immobilized Boc-Lys(ε-NH-Fmoc); see Figure 22. The compounds displayed identical mass spectrometric properties and retention times as the lysine adducts in the pronase digests. The adducts resulting from the glycation pathway are, in this stage of the study, too complex to synthesize.

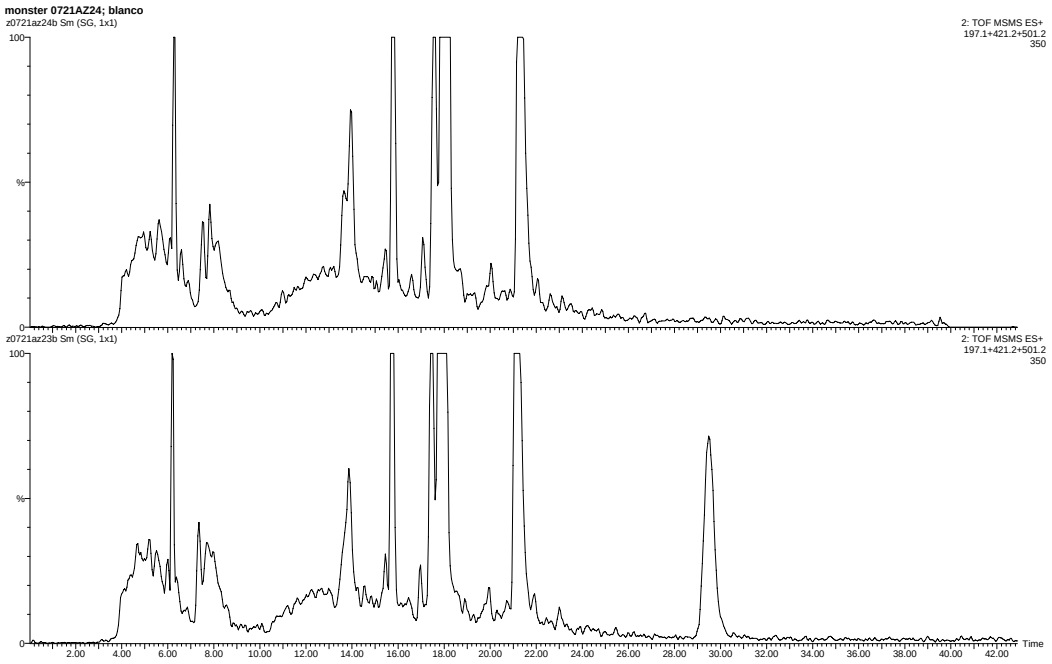

Figure 21. Ion chromatograms for 3-PBA modified lysine (glycation adduct) in a pronase digest of albumin isolated from human plasma that was incubated with 3-PBA glucuronide, that was obtained through enzymatic synthesis. Upper trace, blank plasma; lower trace, exposed plasma. Peak at 29.5 min is peak of interest.

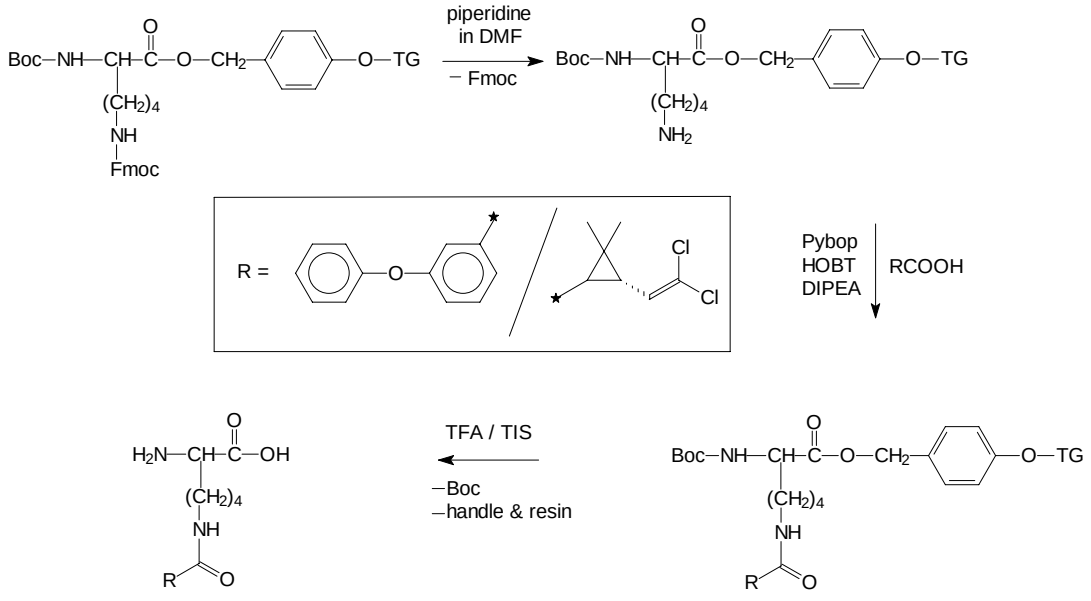

Figure 22. Synthesis scheme for lysine adducts of 3-PBA and Cl_2CA.

Synthesis of [^{14}C] 3-PBA and enzymatic synthesis of [^{14}C] 3-PBA glucuronide

The synthesis of [^{14}C] 3-PBA was performed by using a Grignard reaction with carbon dioxide in order to introduce the [^{14}C] label, basically according to Elliott et al (1976). The synthetic route was first performed by using unlabelled compounds. The synthesis of the labeled material proceeded smoothly and the product displayed similar characteristics as reference 3-PBA (see Figures 23 and 24).

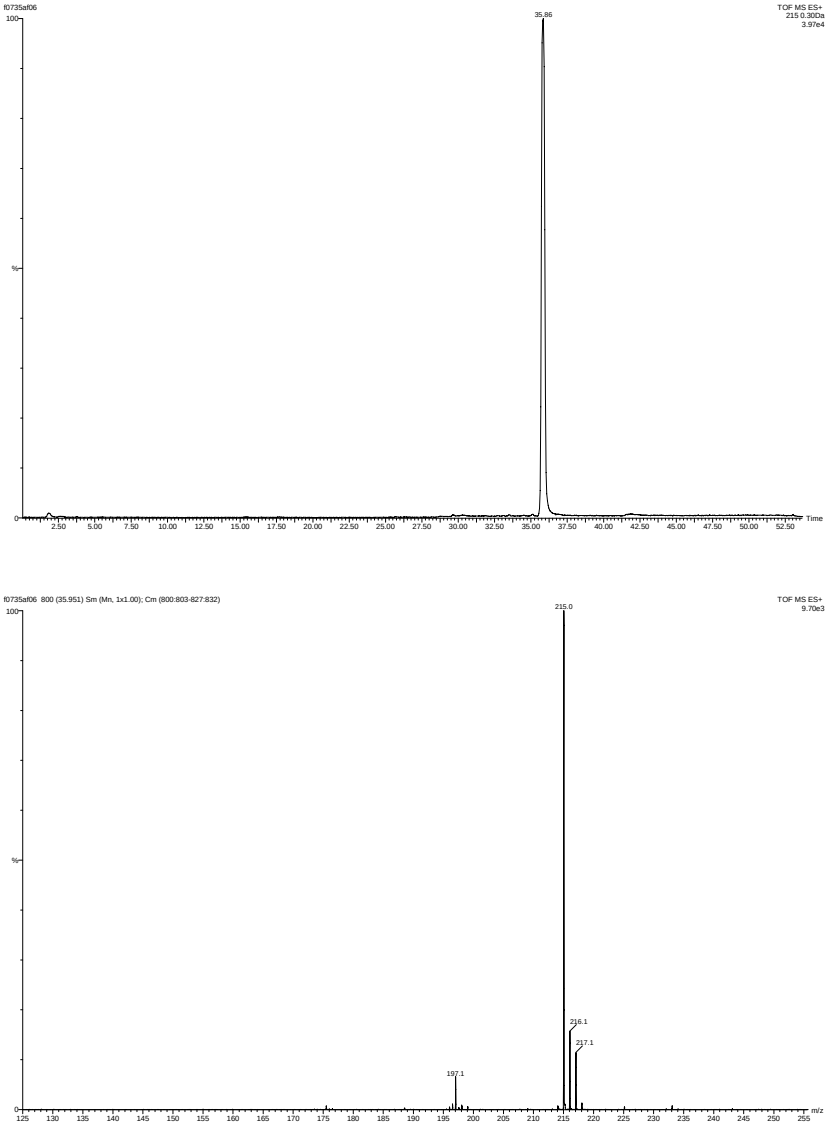

Figure 23. Mass spectrometric analysis of synthetic [^{14}C] 3-PBA. Upper trace: total ion current. Lower trace: mass spectrum, showing the isotope ratio of labeled and unlabeled product.

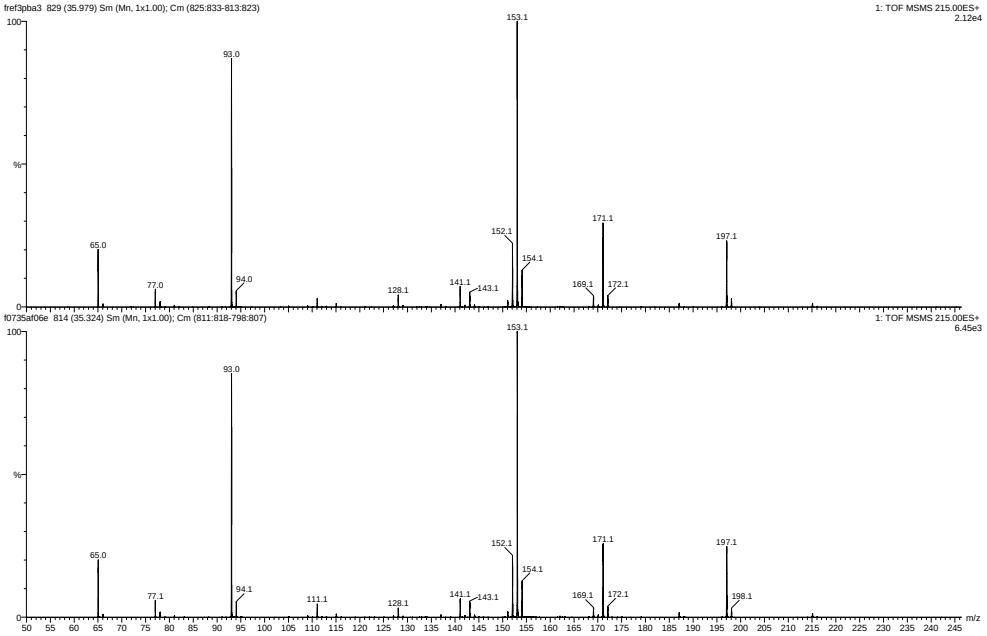

Figure 24. Comparison of commercially available reference standard 3-PBA (upper trace) and [^{14}C] 3-PBA (sp act 6 mCi/mmol), as prepared at TNO.

The enzymatic synthesis of the β-glucuronides was performed according to a literature procedure (Bolze et al., 2002). See Figure 25 for HPLC analysis (radiometric detection) of the crude incubation mixture. The glucuronide was purified by means of reversed phase HPLC. A small part of the purified material was used for thorough mass spectrometric analysis. The ion chromatogram of the isolated product is shown in Figure 26, with the respective mass spectra shown in Figure 27. As was also reported by Bolze et al (2002), the glucuronides rapidly isomerize and consequently various peaks with comparable mass spectra are observed. The mixture as such was used for incubation with plasma.

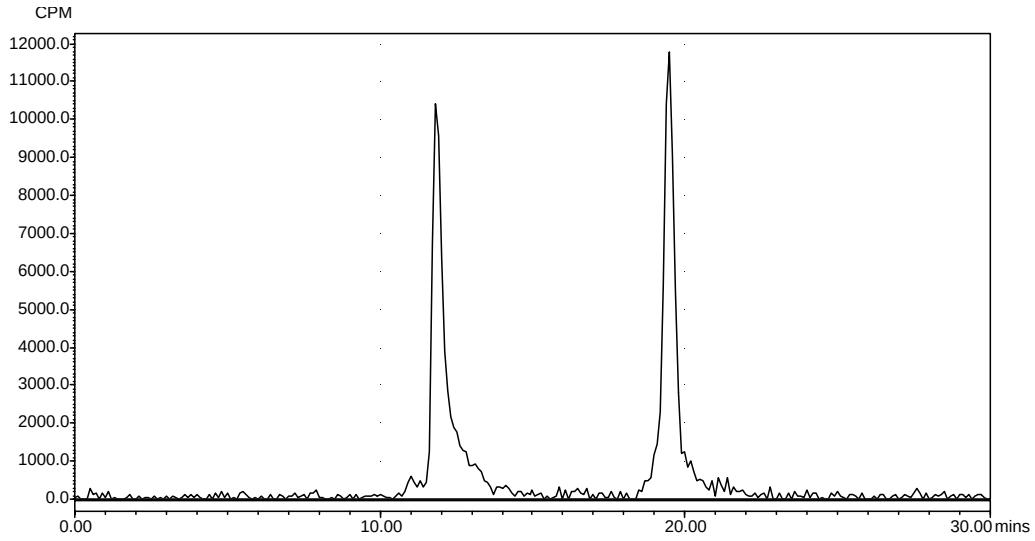

Figure 25. HPLC analysis of biosynthesis incubation mixture for synthesis of [^{14}C] 3-PBA glucuronide from [^{14}C] 3-PBA after 4 h at 37°C. The peak at 20 min represents 3-PBA, the peak at ca 12 min the glucuronide, as was confirmed by mass spectrometry.

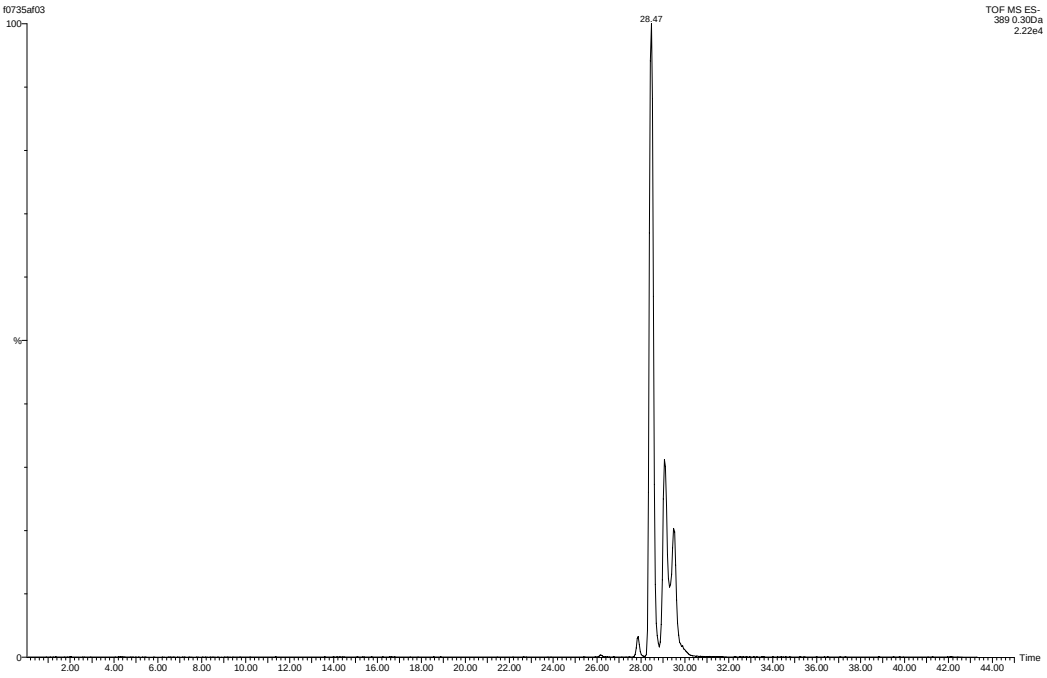

Figure 26. Ion chromatogram of [^{14}C] 3-PBA glucuronide isomers obtained after enzymatic synthesis, after purification by reversed phase HPLC.

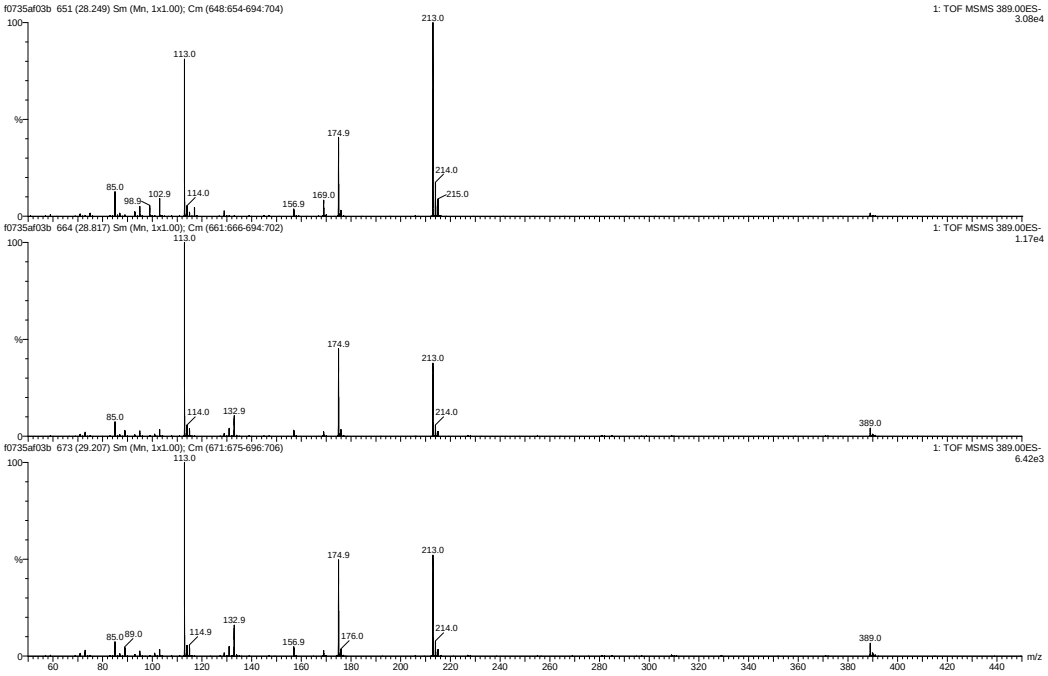

Figure 27. Mass spectra of isomeric [^{14}C] 3-PBA glucuronides obtained after enzymatic synthesis.

Quantitation of binding of [^{14}C] 3-PBA glucuronide in human plasma

The [^{14}C] labelled 3-PBA glucuronides were incubated with plasma for 2 h at 37 ºC. The plasma was subsequently separated into low molecular and high molecular weight material.

Part of the filtrate (Figure 28) and the retentate (Figure 29) of the 10 kD molecular weight cut-off filter were applied to a PD-10 desalting column. The same experiment was applied to plasma exposed to [^{14}C] 3-PBA glucuronide (see Figure 30; 20 micromolar exposure level).

Fractions of 1 mL were collected and analyzed for radioactivity. It was obvious that radioactivity in the retentate fractions coeluted with high molecular material, whereas the radioactivity in the filtrate fractions could be detected in low-molecular weight fractions. A plasma sample applied to a PD-10 column showed radioactivity in both high molecular as low molecular weight fractions.

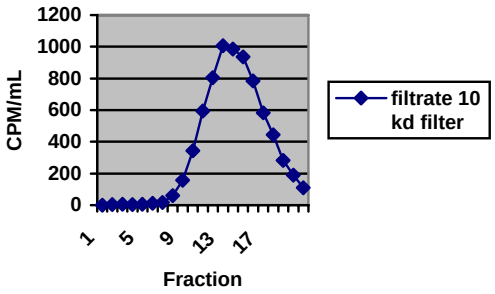

Figure 28. Radioactivity pattern after elution of a 10 kD-filtrate of a plasma sample, exposed to [^{14}C] 3-PBA-glucuronide (20 micromolar), on a PD-10 column.

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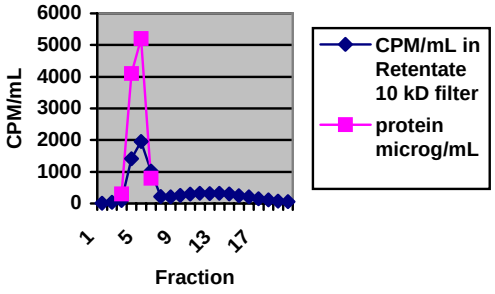

Figure 29. Radioactivity pattern after elution of a 10 kD retentate of a plasma sample exposed to [^{14}C] 3-PBA-glucuronide (20 micromolar), on a PD-10 column.

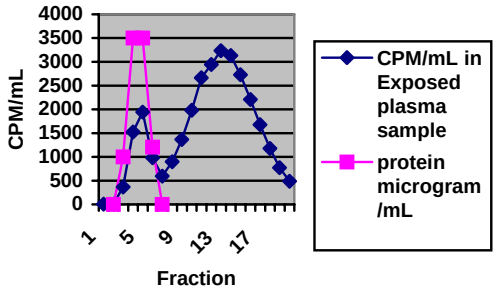

Figure 30. Radioactivity pattern (blue line) after elution of plasma (135 microliter), exposed to 20 micromolar [^{14}C] 3-PBA-glucuronide, on a PD-10 column. The pink line indicates the amount of protein.

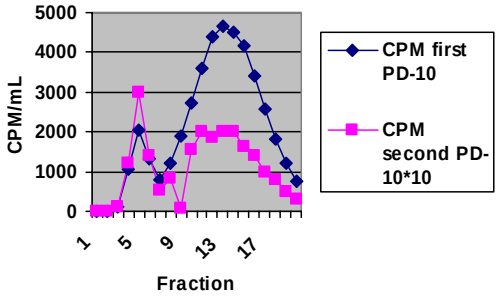

Figure 31. Radioactivity pattern after elution of a plasma sample, exposed to [^{14}C] 3-PBA-glucuronide (20 micromolar) (blue line), on a PD-10 column. The protein containing fractions (3-8) were reapplied to a PD-10 column (pink line), and after elution radioactivity was determined.

One can envisage that 3-PBA and derivatives thereof can also interact with proteins in a non-covalent way. In order to circumvent this issue, the protein-containing fractions were re-eluted over a PD-10 column (see Figure 31). Indeed, part of the radioactivity now eluted in the low-molecular weight fractions, which either points toward non-covalent binding or towards a labile adduct. Therefore, quantitation of binding was studied more careful, e.g. by extensively washing the plasma retentates on a 10 kD molecular weight cut-off filter with 6 M guanidine buffer, followed by a final PD-10 column of the retentate (in PBS), in order to get rid of all non-covalently bound material. It was found that for a 20 micromolar exposure, an average of 1.4 % of the amount of radioactivity added, could be found in the retentate after several washings (ca 5 pmol of [^{14}C] labeled 3-PBA-glucuronide/mg protein) with urea (8M in water). For a 40 micromolar exposure, an average of 1.8 % was found in the retentate (ca 12 pmol of [^{14}C]3-PBA-glucuronide/mg protein) after several washings with urea and 1.2% (ca 9 pmol [^{14}C]3-PBA-glucuronide/mg protein) after several washings with guanidine.HCl (6 M). Bolze et al (*38*) found levels between 0.3 and 5.7 % covalent binding, although he used HSA solutions instead of plasma in his binding studies.

2-D Gel electrophoresis experiments with radioactively labeled 3-PBA glucuronide

Protein binding of 3-PBA glucuronide was studied in more detail by using 2-D gel-electrophoresis. Plasma samples were exposed to the radioactively labeled glucuronide. Subsequently, the plasma proteins were separated by 2-D gel electrophoresis, and subsequently radioactivity was determined by means of autoradiography (see Figure 32). Apparently, albumin is the major site of modification.

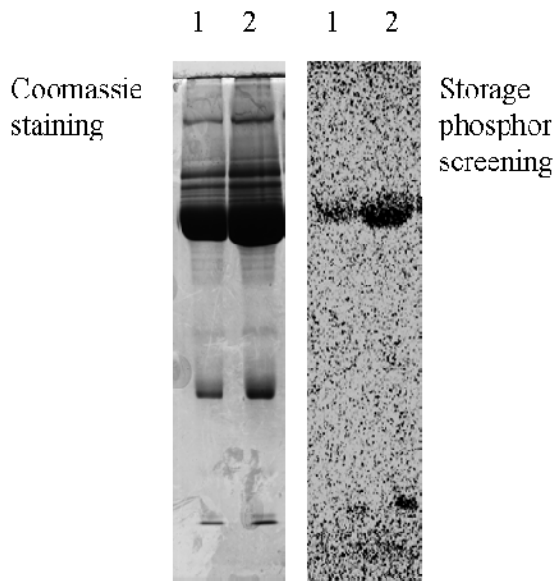

Figure 32. 2-D gel electrophoresis of human plasma incubation mixture with [^{14}C]-labelled 3-PBA glucuronide. Left: protein visualization by coomassie staining. Right: radioactivity determination by storage phosphor screening.

In case of liver homogenates, almost all radioactivity was found in low-molecular material (see Figure 33). Human plasma was included as a control sample. Again, in case of plasma the majority of the radioactivity found was bound to high molecular material, probably albumin. In view of the experiments in which the degree of binding was carefully quantified (see above), the non-covalent binding is apparently strong enough to survive 2-D gel electrophoresis conditions. Probably, the [^{14}C]-3-PBA, resulting from hydrolysis of the glucuronide diffuses into the electrophoresis buffer. The fact that binding to liver proteins is much less predominant than in case of plasma might be explained by the fact that the 3-PBA glucuronide hydrolyses more rapidly in liver homogenate (spontaneous or enzyme-induced, e.g., by β-glucuronidase), or is preferably bound to low-molecular compounds. Evidently, plasma albumin is a very good scavenger of the reactive 3-PBA glucuronide. With more sensitive radioactivity detection methods, target proteins might also be revealed in liver homogenates.

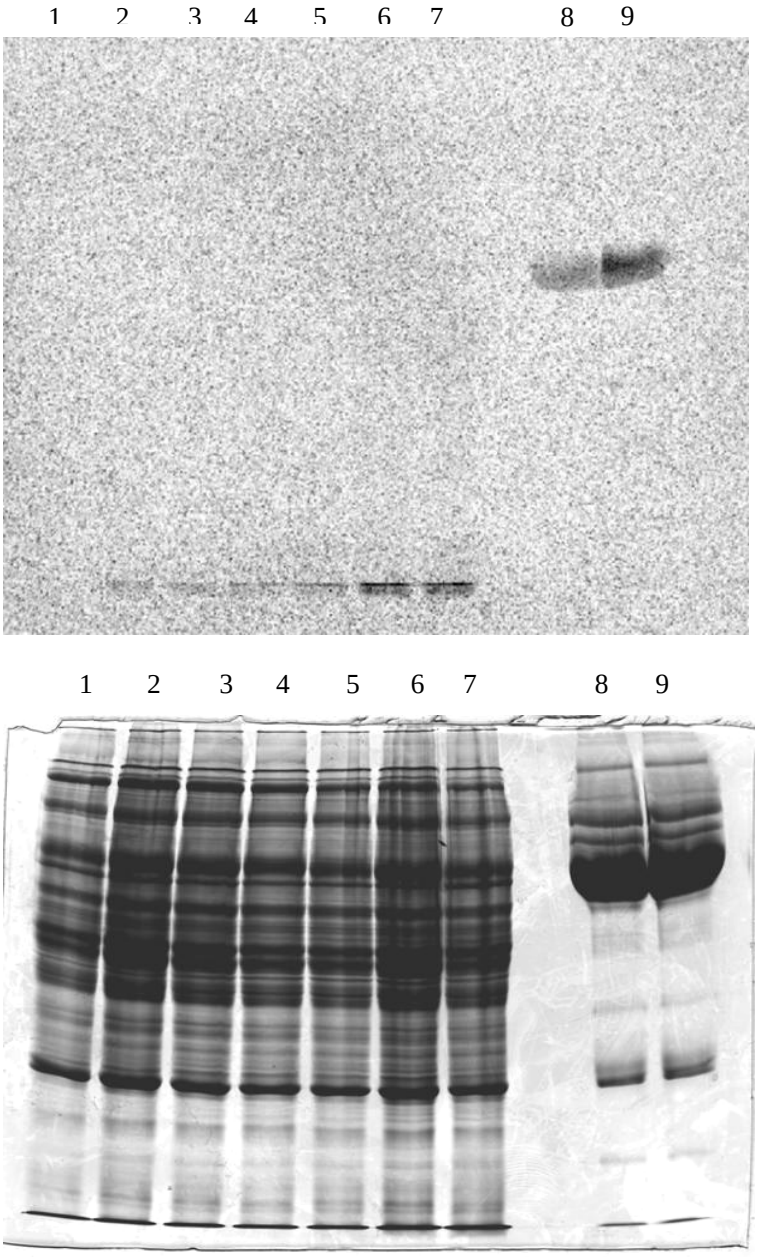

Figure 33. 2-D gel electrophoresis of rhesus monkey liver homogenate incubation mixture with [^{14}C]-labelled 3-PBA glucuronide at various concentrations. Lane 1: unexposed liver homogenate sample. Lane 2 and 3: 0.1 mM; Lanes 4 and 5: 0.5 mM; Lanes 6 and 7: 1.0 mM. Lanes 8 and 9: human plasma control samples, incubated with 0.1 (lane 8) and 0.5 mM (lane 9) [^{14}C]-labelled 3-PBA glucuronide. Lower picture: protein visualization by coomassie staining. Upper picture: radioactivity determination by storage phosphor screening.

Pilot animal experiment

We were anxious to find out whether we could detect one of the 3-PBA – lysine adducts after in vivo exposure to permethrin. Therefore, a guinea pig was exposed to a high dose of permethrin (20 mg/kg, s.c.) and blood samples were taken at various time points. In this case, the entire plasma sample was digested with pronase; the albumin isolation step was skipped, because of the limited blood sample sizes. The adduct resulting from the glycation reaction could not be detected. However, the acyl adduct between lysine and 3-PBA could be detected even in a sample taken 22 days after injection of permethrin. Most of the adduct could be found 3 days after injection of permethrine (although no internal standard was used. To find out whether adduct formation was not caused by bioactivation we incubated plain 3-PBA with guinea pig blood in vitro at high levels (0.3 and 1.2 mg/mL blood). Incubation was performed during 2 hours at 37 °C. The work-up procedure was exactly as performed for the in vivo samples. No acyl adduct formation could be observed in these samples. In view of the fact that only the acyl adduct could be detected, we considered that this animal experiment was not representative for a human exposure.

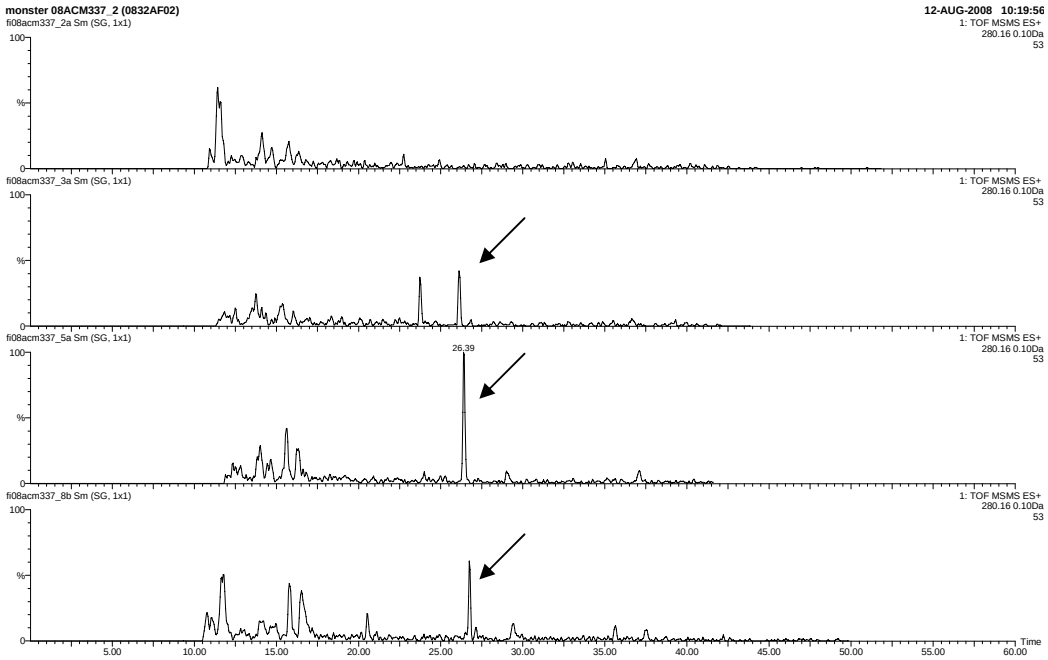

Figure 34. Ion chromatogram of 3-PBA-lysine acyl adduct (*m/z* 290; MH^+; see arrow) in a pronase digest of whole plasma, obtained from a guinea pig that had been exposed to permethrin (20 mg/kg, s.c.). From top to bottom: control sample (before exposure), 2 days, 4 days, and 22 days after the exposure.

Analysis of human plasma samples from volunteers after wearing permethrin-treated suits

A total of 33 blood samples (3 samples from 11 participants; 10 ml in EDTA tubes) were collected. The blood samples were separated into plasma and red blood cells according to well-established procedures, and shipped to TNO. An aliquot of each plasma sample was directly digested with pronase (as in case of the animal blood samples) and the digests were analyzed by LC-tandem MS analyses. No indications were found for the presence of 3-PBA lysine acyl adducts, or of 3-PBA lysine glycation adducts. Apparently, the exposure level was too low to result in detectable levels of 3-PBA adducts. We will repeat the work-up of a number of plasma samples by including an albumin isolation step, and analyze the digests.

General conclusion/future prospects

In conclusion, the reaction of the permethrin-derived β-glucuronides of 3-PBA and Cl_2CA with model peptide compounds has been shown to result in the formation of both ε-N-lysinyl adducts formed by transacylation, as well as ε-N-lysinyl adducts formed by the glycation mechanism (see Noort et al., 2008). When incubation experiments with human plasma were carried out, adduct formation could be observed with albumin after mass spectrometric analysis of trypsin and pronase digests of albumin. Pronase digestion of albumin isolated from exposed plasma samples resulted in the formation of the various individual ε-NH_2-modified lysine derivatives, which can be analyzed conveniently by mass spectrometry. These adducts had favourable chromatographic and mass spectrometric properties. With regard to the toxicological relevance, it seems that in liver protein binding is less predominant than in plasma. We were not able to identify modified liver proteins. The lysine – 3-PBA acyl adduct could be detected in a pronase digest of whole plasma of an exposed guinea pig, even after 22 days after the intoxication. In plasma samples of human volunteers from a USARIEM study who had been wearing permethrin-treated suits, no permethrin-derived adducts could be detected by mass spectrometric analysis after pronase digestion of whole plasma. Although it seems that the 3-PBA glucuronide adducts are not being formed in such amounts that they can serve as biomarkers for permethrin exposure within the framework of biomonitoring studies, the current large scale use of permethrin warrants further investigations towards the toxicological relevance of protein binding by the reactive glucuronide metabolites.

Also, the mass spectrometric method used for analysis of the lysine adduct can be further optimized; it is envisaged that GC-MS analysis of the lysine adducts, after derivatization, might give lower detection limits. In addition, because of the occurrence of an intermediate 3-phenoxybenzaldehyde during permethrin metabolism, it should be investigated whether 3-phenoxybenzaldehyde-derived adducts also occur.

V APPENDIX

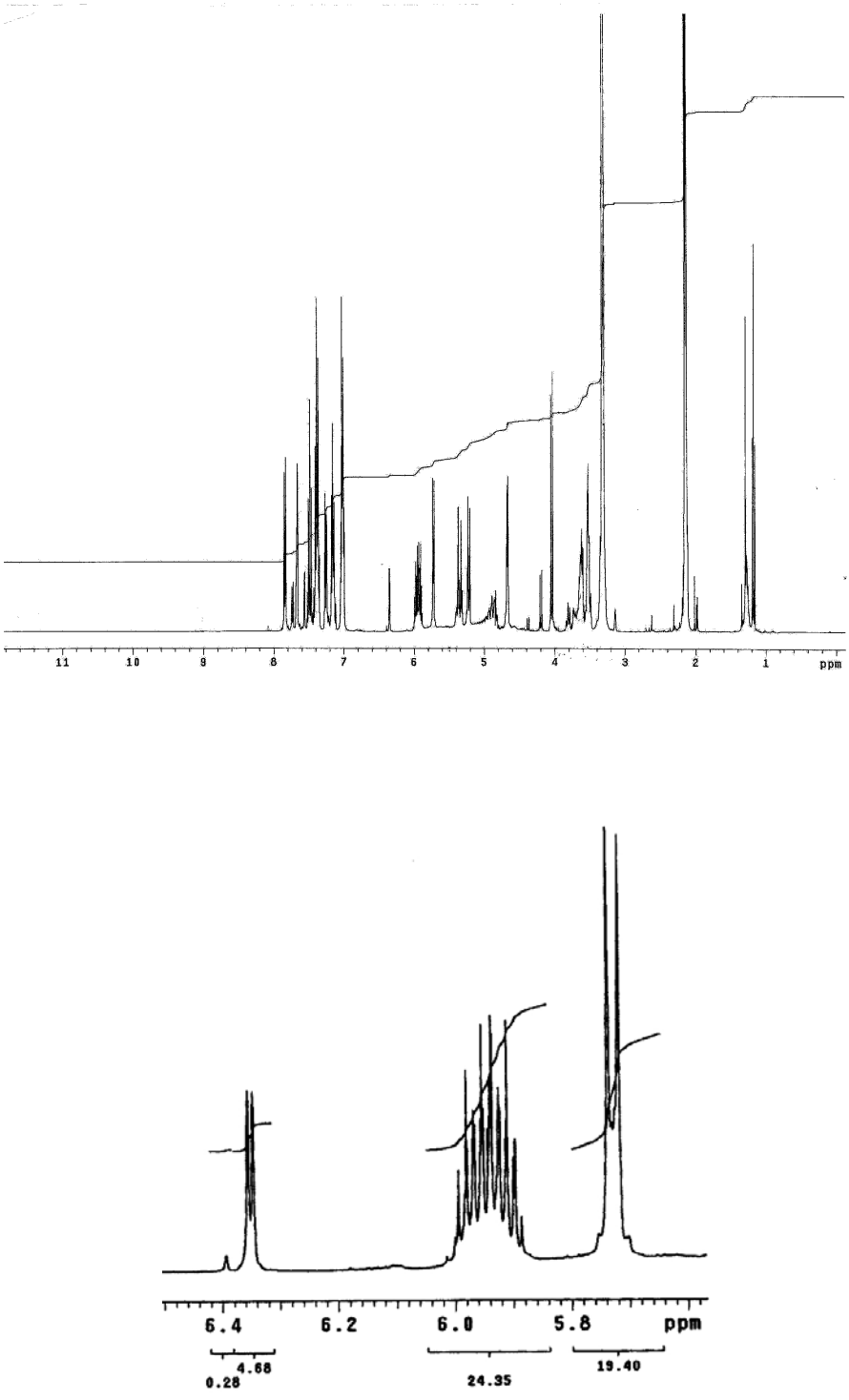

Figure 1A. ^{1}H-NMR spectrum of anomeric mixture of allyl 1-O-(3-phenoxybenzoyl)-D-glucopyranuronate (2). Upper panel: entire 400 MHz 1H-NMR spectrum. Lower panel: part of the spectrum, showing the H1-α around 6.4 and H1-β around 5.7. The multiplet around 5.9 – 6.0 is part of the allyl group.

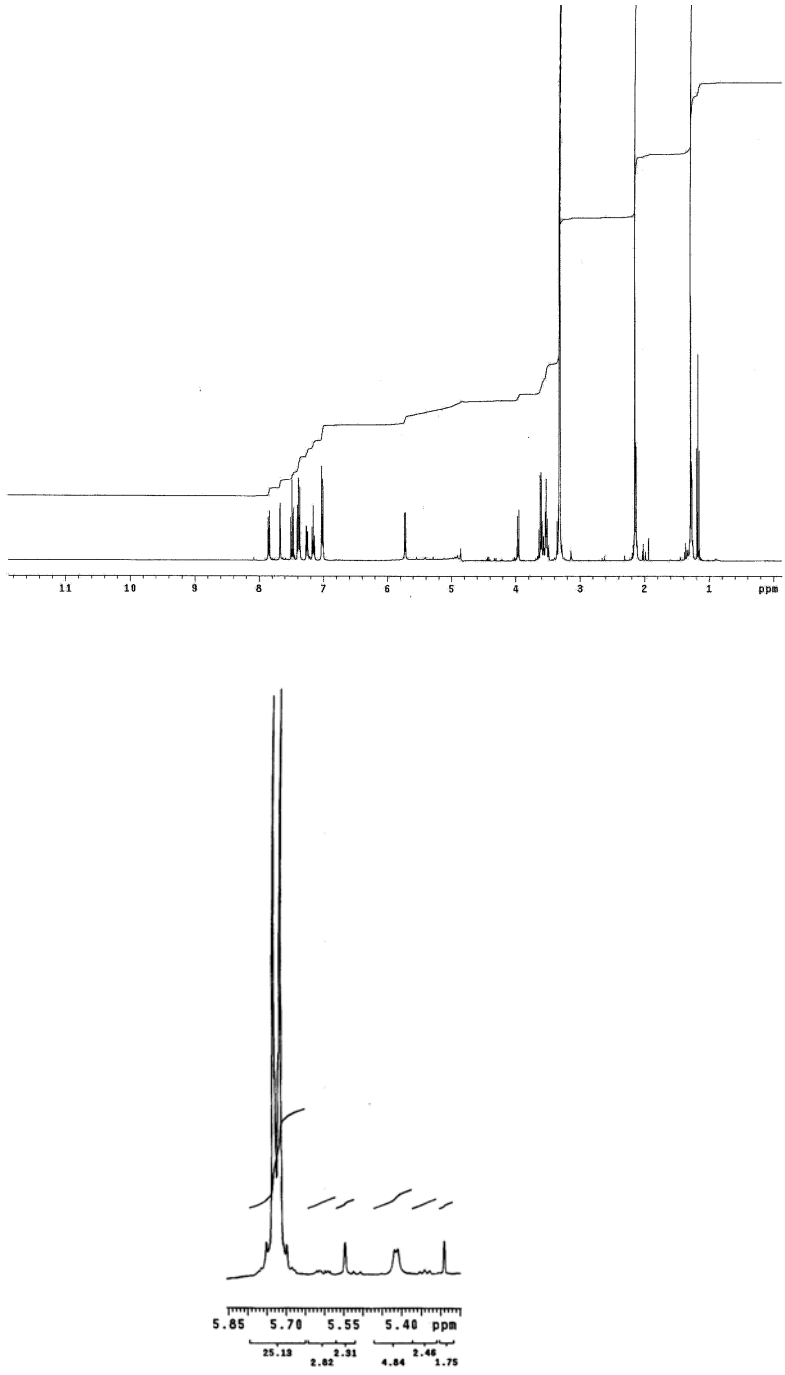

Figure 2A. [1]H-NMR spectrum of anomeric mixture of 1-O-(3 phenoxy-benzoyl)-D-glucopyranuronate (3; 3-PBA-glucuronide). Upper panel: entire [1]H-NMR spectrum. Lower panel: part of the 1H-NMR spectrum, showing the characteristic doublet of H1-β around 5.7.

VI KEY RESEARCH ACCOMPLISHMENTS

1. The synthesis of the β-acyl glucuronide of the permethrin-derived metabolite 3-PBA has been successfully accomplished, after exploring two different synthetic routes.
2. The synthesis of the acyl glucuronide of the permethrin-derived metabolite Cl_2CA has been accomplished, although as yet the α- and β-anomers cannot be separated.
3. The reaction of 3-PBA glucuronide with glutathione has been shown to result in an (unstable) S-acylated adduct.
4. The reaction of 3-PBA glucuronide with model compounds has been shown to result in the formation of both adducts formed by transacylation, as well as adducts formed by the glycation mechanism.
5. For Cl_2CA glucuronide, only adducts with glutathione could be detected, derived from transacylation. No adducts could be observed after incubation of Cl_2CA glucuronide with amino-containing model compounds
6. A method was developed for enzymatic synthesis of 3-PBA glucuronide and Cl_2CA glucuronide.
7. The enzymatically obtained glucuronides of 3-PBA and Cl_2CA gave also rise to adduct formation with proteins.
8. A method was developed for release and subsequent mass spectrometric analysis of adducts of 3-PBA glucuronide and Cl_2CA glucuronide to lysine residues in proteins.
9. The synthesis of lysine adducts of 3-PBA and Cl_2CA glucuronide, resulting from the transacylation mechanism, has been accomplished. These compounds can be used as reference compounds for eventual biomonitoring purposes.
10. The synthesis of [^{14}C] labelled 3-PBA has been accomplished.
11. The synthesis of [^{14}C] labelled 3-PBA glucuronides, either with the radioactive label in the 3-PBA moiety or in the glucuronide moiety, has been accomplished.
12. Quantitation of binding of [^{14}C] labelled 3-PBA glucuronide to plasma proteins has been undertaken. The binding studies were significantly thwarted by apparently strong non-covalent interactions of the 3-PBA derivatives with proteins.
13. From a 2-D gel electrophoresis experiment with [^{14}C]labelled 3-PBA glucuronide (with a high specific activity) and human plasma, it could be derived that the major binding site in plasma was human serum albumin.
14. In liver homogenate protein binding of 3-PBA glucuronide seems much less predominant than in case of plasma.
15. When a guinea pig was exposed to a high dose of permethrin, the lysine – 3-PBA acyl adduct could be detected in a pronase digest of whole plasma of the exposed animal, even after 22 days after the intoxication.

16. In plasma samples of human volunteers from a USARIEM study who had been wearing permethrin-treated suits, no permethrin-derived adducts could be detected after pronase digestion of whole plasma.

VII REPORTED OUTCOMES

Publications

Biomonitoring of exposure to permethrin based on adducts to proteins
D. Noort, A. van Zuylen, A. Fidder and A.G. Hulst
In 'Defence against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures', pp. 24-1 – 24-10.
Meeting proceedings RTO-MP-HFM-149, paper 24. Available from http://www.rto.nato.int

Human exposure biomarkers: permethrin as a military-relevant model
K.-G. Mross, G.E. Adam, D. Noort, and J. Zimmer.
In 'Biotechnologies for assessment of toxic hazards in operational environments', pp. 7-1 – 7-20
Final report of HFM-057/RTG-009. Available from http://www.rto.nato.int

Protein adduct formation by glucuronide metabolites of permethrin
D. Noort, A. van Zuylen, A. Fidder, B. van Ommen and A.G. Hulst
Chem. Res. Toxicol. 21, 1396-1406 (2008)

Presentations

Generic assays for exposure biomonitoring of alkylating agents
NATO TG-009
Natick, MA, USA, October 2005
Presented by D. Noort

Persistent biomarkers of exposure
2nd International Workshop on Environmental and Health Hazards,
The Hague, The Netherlands, April 5&6, 2006
Presented by D. Noort

Persistent biomarkers of exposure of potentially neurotoxic compounds
23rd International Neurotoxicology Conference
Little Rock, USA, September 17-21, 2006
Presented by D. Noort

Biomonitoring of exposure to permethrin based on adducts to proteins

NATO Human Factors & Medicine Panel Symposium ‘Defense against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures’

October 8-10, 2007, Edinburgh, UK

Presented by D. Noort

VIII CONCLUSIONS

1. β-Acyl glucuronides of permethrin metabolites are intrinsically reactive compounds which can be obtained by enzymatic or organic synthesis.
2. Adduct formation of the intrinsically reactive acyl glucuronide of the permethrin metabolite 3-PBA with various model amino acid and peptide model compounds has been assessed. Thus far, adduct formation of the similar acyl glucuronide of the permethrin metabolite Cl_2CA could only be seen with glutathione.
3. The reaction of the permethrin-derived β-glucuronides of 3-PBA and Cl_2CA with proteins has been shown to result in the formation of both ε-N-lysinyl adducts formed by transacylation, as well as ε-N-lysinyl adducts formed by the glycation mechanism.
4. When incubation experiments with human plasma were carried out, adduct formation could be observed with albumin after mass spectrometric analysis of trypsin and pronase digests of albumin. This was the case for glucuronides prepared either by organic synthesis or by enzymatic biosynthesis.
5. Pronase digestion of albumin isolated from exposed plasma samples resulted in the formation of the various individual ε-NH_2-modified lysine derivatives, which can be analyzed conveniently by mass spectrometry. These adducts had favourable chromatographic and mass spectrometric properties.
6. Protein binding studies were thwarted by non-covalent interactions with (radioactively labeled) 3-PBA glucuronide or derivatives thereof; it was estimated that at least 1-2% of 3-PBA glururonide becomes covalently bound to proteins.
7. From a 2-D gel electrophoresis experiment with [^{14}C]labelled 3-PBA glucuronide (with a high specific activity) and human plasma, it could be derived that the major binding site in plasma was human serum albumin.
8. The fact that binding to liver proteins is much less predominant than in case of plasma might be explained by the fact that the 3-PBA glucuronide hydrolyses more rapidly in liver homogenate (spontaneous or enzyme-induced, e.g., by β-glucuronidase), or is preferably bound to low-molecular compounds.
9. The lysine – 3-PBA acyl adduct could be detected in a pronase digest of whole plasma of a permethrin-exposed guinea pig, even after 22 days after the intoxication.
10. In plasma samples of human volunteers from a USARIEM study who had been wearing permethrin-treated suits, no permethrin-derived adducts could be detected by mass spectrometric analysis after pronase digestion of whole plasma.
11. Although it seems that the 3-PBA glucuronide adducts are not being formed in such amounts that they can serve as biomarkers for permethrin exposure within the framework of biomonitoring studies, the current large scale use of permethrin

warrants further investigations towards the toxicological relevance of protein binding by the reactive glucuronide metabolites.

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X BIBLIOGRAPHY OF PUBLICATIONS AND MEETING ABSTRACTS

Publications

Biomonitoring of exposure to permethrin based on adducts to proteins
D. Noort, A. van Zuylen, A. Fidder and A.G. Hulst
In 'Defence against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures', pp. 24-1 – 24-10.
Meeting proceedings RTO-MP-HFM-149, paper 24.
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Human exposure biomarkers: permethrin as a military-relevant model
K.-G. Mross, G.E. Adam, D. Noort, and J. Zimmer.
In 'Biotechnologies for assessment of toxic hazards in operational environments', pp. 7-1 – 7-20
Final report of HFM-057/RTG-009.
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Protein adduct formation by glucuronide metabolites of permethrin
D. Noort, A. van Zuylen, A. Fidder, B. van Ommen and A.G. Hulst
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Meeting Abstracts

Persistent biomarkers of exposure
D. Noort
Abstract book 2nd International Workshop on Environmental and Health Hazards,
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Persistent biomarkers of exposure of potentially neurotoxic compounds
D. Noort
Abstract book 23rd International Neurotoxicology Conference
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Biomonitoring of exposure to permethrin based on adducts to proteins
D. Noort
Abstract book for NATO Human Factors & Medicine Panel Symposium 'Defense against the effects of chemical hazards. Toxicology, diagnosis, and medical countermeasures'
October 8-10, 2007, Edinburgh, UK.

XI LIST OF PERSONNEL RECEIVING PAY UNDER THIS COOPERATIVE AGREEMENT

Dr. D. Noort
Dr. B.L.M. van Baar
Mr. A. Fidder
Mr. A.G. Hulst
Mr. S.H. van Krimpen
Mrs. A. Van Zuylen